UNIVERSIDAD DE CÓRDOBA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

NUEVAS ESTRATEGIAS EN LA PREPARACIÓN DE LA MUESTRA Y EN EL DESARROLLO DE PLATAFORMAS PARA EL ANÁLISIS METABOLÓMICO ORIENTADO Y GLOBAL

NEW STRATEGIES ON SAMPLE PREPARATION AND ON PLATFORMS DEVELOPMENT FOR TARGETED AND UNTARGETED METABOLOMICS ANALYSIS

María del Mar Delgado Povedano

Directores: María Dolores Luque de Castro y Feliciano Priego Capote

Programa de doctorado de Química Fina

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MARÍA DEL MAR DELGADO POVEDANO

TESIS DOCTORAL
CÓRDOBA, 2019

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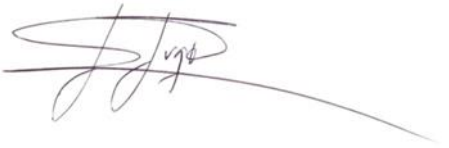

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Trabajo presentado para optar al grado de
Doctor en Ciencias, Sección Química

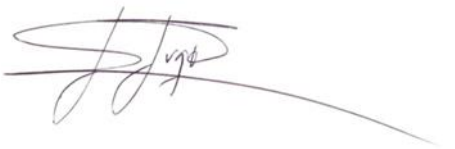
Fdo. María del Mar Delgado Povedano
Licenciada en Química

María Dolores Luque de Castro, Catedrática Emérita, y **Feliciano Priego Capote**, Profesor Titular, ambos del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por la Licenciada en Química **María del Mar Delgado Povedano,** con el título "**Nuevas estrategias en la preparación de la muestra y en el desarrollo de plataformas para el análisis metabolómico orientado y global**",

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, a 21 de octubre de 2019.



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Fdo. Feliciano Priego Capote

Mediante la defensa de esta Memoria se pretende optar a la mención de **Doctorado Internacional**, habida cuenta de que la doctoranda reúne los requisitos exigidos para tal mención, a saber:

1. Informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:
   - Prof. Dr. André De Villiers, Department of Chemistry, Stellenbosch University, Stellenbosch, South Africa.
   - Prof. Dr. José Luis Capelo Martínez, Department of Chemistry, University Nova of Lisbon, Lisbon, Portugal.
2. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país:
   - B.Sc. Ph.D. Tim Causon, Department of Chemistry, Division of Analytical Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria.
3. La exposición y la defensa de parte de esta Tesis se realizarán en una lengua diferente a la materna: inglés.
4. Estancia de tres meses en un centro de investigación de otro país:
   - Department of Chemistry, Division of Analytical Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria, bajo la supervisión del Prof. Dr. Stephan Hann.

TÍTULO DE LA TESIS: Nuevas estrategias en la preparación de la muestra y en el desarrollo de plataformas para el análisis metabolómico orientado y global

DOCTORANDA: María del Mar Delgado Povedano

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda María del Mar Delgado Povedano, primero como estudiante y actualmente como investigadora, ha mostrado siempre un gran interés por todas las ramas de la ciencia en general y de la química analítica en particular. En el campo concreto de la metabolómica, su interés en las áreas vegetal y clínica condujo a la selección en ambas de temas de impacto que requirieran una investigación pionera y abriera nuevas líneas en las que avanzar en esta disciplina. Una vez desarrollados los estudios previstos, los resultados se han dividido en tres bloques: bibliografía, metabolómica vegetal y metabolómica clínica.

-Un primer bloque (Bloque A) pone de manifiesto la formación adquirida por la doctoranda en el manejo de la información bibliográfica, especialmente orientada a la preparación de la muestra, con la publicación de un capítulo de libro sobre extracción asistida por ultrasonidos (US), una revisión bibliográfica sobre el uso de los US en metabolómica y otra sobre los contravertidos efectos de los US en la actividad enzimática y en los propios biocatalizadores, que constituyen los Capítulos I, II y III, respectivamente, de la Memoria de Tesis. La continua actualización de la literatura científica mantenida a lo largo de desarrollo de la Tesis ha dado lugar también a otras publicaciones, que se recogen en la Memoria como anexos; todas ellas ponen de manifiesto su amplia formación en este aspecto.

-El segundo bloque de la Memoria (Bloque B) comienza con el Capítulo IV, que aprovecha la experiencia adquirida sobre US para iniciar una línea de investigación sobre la influencia de esta energía en los procesos de hidrólisis enzimática. El estudio se centra en la oleuropeína como materia prima obtenida de extractos de hojas de olivo para la producción de la correspondiente aglicona. Otra línea de investigación de interés se abre en el Capítulo V con la identificación de 123 metabolitos (55 identificados por primera vez) existentes en los extractos enzimáticos acuosos de la seta comestible *Agaricus bisporus*, ya que esta seta ha mostrado su capacidad de inhibición de la proteasa NS3 del virus de la hepatitis C, de ahí el enorme interés de la exhaustiva identificación realizada. Una plataforma constituida por un cromatógrafo de líquidos y un detector de masas de tiempo de vuelo (LC–QTOF MS/MS) constituye la herramienta analítica más adecuada para este tipo de estudios. De no menor interés, dado el incremento en los últimos años del uso farmacológico del cannabis, es la investigación que se recoge en el Capítulo VI de la Memoria. Dos plataformas analíticas basada una de ellas en un cromatógrafo de gases y un detector de masas de tiempo de vuelo (GC–TOF/MS) y la otra en LC–QTOF MS/MS han hecho posible la identificación de 169 compuestos (cannabinoides y terpenoides), el estudio de 17 variedades de la planta y la comparación relativa de los compuestos en

función de su desarrollo en invernadero o en campo. Información clave la que proporciona este estudio, dado el interés creciente de muchos países en legalizar el uso total o parcial de esta planta.

Los Bloques C y D tienen como común denominador, además de la metabolómica, la muestra clínica que se estudia: el sudor.

-En el Bloque C el uso de una plataforma analítica basada en LC y un detector de triple cuadrupolo (QqQ) dio lugar al único método de análisis metabolómico dirigido para la cuantificación de aminoácidos en sudor (Capítulo VII). El papel clave que tienen estos compuestos en la diagnosis y tratamiento de diferentes enfermedades les hace cobrar una especial relevancia en el caso de fluidos biológicos relativamente poco explotados, como es el caso del sudor.

Los desarrollos de análisis metabolómico no dirigidos se focalizaron en el estudio de los diferentes tipos de muestreadores y muestras de sudor: muestreo en diferentes partes del cuerpo de sudor después de ejercicio moderado comparado con sudor obtenido en reposo mediante muestreo pasivo, tal como se recoge en el Capítulo VIII. Las estrategias de derivatización para la conversión de los metabolitos en compuestos volátiles suponen una aportación clave en esta investigación. El muestreo de sudor seco constituye una novedad digna de destacarse, ya que requirió el estudio de diferentes soportes impregnados de diferentes disolventes para la recogida de las muestras y su comparación para la selección del conjunto óptimo. El uso de las plataformas GC–MS y LC–MS/MS para la identificación de los diferentes metabolitos y la comparación con los existentes en sudor fresco permitió delimitar las posibles aplicaciones de cada tipo de sudor, tal como se recoge en el Capítulo IX.

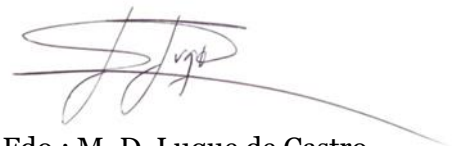
-El Bloque D tiene un perfil aplicado, ya que se dedica a la mejora los métodos para abarcar un mayor número de metabolitos y, por tanto, dar lugar a un mayor número de posibilidades de aplicación (Capítulo X); lo que proporciona una base sólida para la proposición de metabolitos del sudor como biomarcadores de cáncer de pulmón (Capítulo XI). El uso de herramientas quimiométricas actuales, como el programa PanelomiX, ha permitido obtener dos paneles de metabolitos que reducen tanto el número de falsos negativos como de falsos positivos y proporcionan valores de especificidad y de sensibilidad del 95%.

Por todo lo anterior, consideramos que la investigación desarrollada por María del Mar Delgado Povedano y recogida en esta Memoria reúne la originalidad, innovación y calidad requeridas.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 21 de octubre de 2019

Firmas de los directores



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INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES DE LA TESIS

TÍTULO DE LA TESIS: NUEVAS ESTRATEGIAS EN LA PREPARACIÓN DE LA MUESTRA Y EN EL DESARROLLO DE PLATAFORMAS PARA EL ANÁLISIS METABOLÓMICO ORIENTADO Y GLOBAL

DOCTORANDA: MARÍA DEL MAR DELGADO POVEDANO

Publicaciones	**FI (JCR)** (5 years)	**Decil/Cuartil**
Study of sample preparation for quantitative analysis of amino acids in human sweat by liquid chromatography-tandem mass spectrometry. ***Talanta*** 2016; 146; 310–317.	3.841	Q1 9/76 Analytical Chemistry
Development of a method for enhancing metabolomics coverage of human sweat by gas chromatography-mass spectrometry in high resolution mode. ***Anal. Chim. Acta*** 2016; 905; 115–125.	4.849	D1 7/76 Analytical Chemistry
Tentative identification of the composition of *Agaricus bisporus* aqueous enzymatic extracts with antiviral activity against HCV: A study by liquid chromatography-tandem mass spectrometry in high resolution mode. ***J. Funct. Foods*** 2016; 24; 403–419.	3.460	Q1 18/130 Food Science & Technology
Recent advances in human sweat metabolomics for lung cancer screening. ***Metabolomics*** 2016; 12; 166.	3.933	Q2 45/138 Endocrinology & Metabolism
Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts. ***Food Chem.*** 2017; 220; 282–288.	4.879	D1 7/133 Food Science & Technology
Metabolomics analysis of human sweat collected after moderated exercise. ***Talanta*** 2018; 177; 47–65.	4.322	Q1 16/117 Analytical Chemistry
Untargeted characterization of extracts from *Cannabis sativa* L. cultivars by gas and liquid chromatography coupled to mass spectrometry in high resolution mode. ***Talanta*** (https://doi.org/10.1016/j.talanta.2019.120384).	4.322	Q1 16/117 Analytical Chemistry
Dry sweat as sample for metabolomics analysis. ***Talanta*** (https://doi.org/10.1016/j.talanta.2019.120428).	4.322	Q1 16/117 Analytical Chemistry
Ultrasound-assisted analytical emulsification-extraction. ***Trends Anal. Chem.*** 2013; 45; 1–13.	7.311	D1 2/76 Analytical Chemistry

Ultrasound-assisted extraction and in situ derivatization. ***J. Chromatogr. A*** 2013; 1296; 226–234.	4.339	D1 6/76 Analytical Chemistry
Ultrasound: A subexploited tool for sample preparation in metabolomics. ***Anal. Chim. Acta*** 2014; 806; 74–84.	4.667	D1 5/74 Analytical Chemistry
A review on enzymes and ultrasound: A controversial but fruitful relationship. ***Anal. Chim. Acta*** 2015; 889; 1–21.	4.841	Q1 8/75 Analytical Chemistry
The "in medium virtus" assessment of green analytical chemistry. ***Current Opinion in Green and Sustainable Chemistry*** 2019; 19; 8–14.	1.032*	Q1 65/437 Chemistry (miscellaneous)

*SCImago Journal Rank; FI, factor de impacto; JCR, Journal Citation Reports; Q1, first quartile; D1, first decile.

Resumen

1. Motivación de la tesis

La hipótesis que precedió al establecimiento de los objetivos sobre los que se planteó la investigación fue la existencia de un gran número de retos en metabolómica vegetal y animal de los que se requiere su abordaje. Por tanto, el objetivo básico abarcó ambas áreas de la metabolómica: la vegetal y la animal. Los tres pilares en los que se soportó la investigación desarrollada fueron el diseño de la plataforma analítica apropiada para cada caso, la supervisión por investigadores con reconocida experiencia en metabolómica y la formación y capacidad de la doctoranda para aceptar y desarrollar los retos planteados.

2. Contenido de la investigación

La investigación realizada ha implicado:

-Un estudio en profundidad de la bibliografía relacionada con el trabajo planteado, complementado con una actualización continua durante todo el desarrollo de la tesis para mantener la investigación en primera línea. Se prestó especial atención a temas relacionados con la preparación de la muestra y la metabolómica, incluyendo una herramienta comúnmente utilizada por el grupo de investigación al que pertenece la doctoranda: los ultrasonidos (US) usados como energía auxiliar para favorecer etapas analíticas. De hecho, la extracción asistida por US (USAE) [1], los US y la metabolómica [2] y el controvertido efecto de los US en la actividad enzimática [3] han dado lugar a tres publicaciones que constituyen la Sección A de la tesis.

-La hidrólisis de la oleuropeína en extractos de hojas de olivo acelerada por diferentes hidrolasas y por la acción de los US (USAEH) [4] (que abre la puerta al complejo mundo del binomio enzimas–US), el uso de una plataforma basada en cromatografía líquida y espectrometría de masas en tándem (LC–QTOF MS/MS) que proporcionó los datos para la identificación tentativa de 123 metabolitos de extractos que habían producido la inhibición de la proteasa NS3 del virus de la hepatitis-C [5] constituyen logros plasmados en publicaciones. Otro de los logros fue la exhaustiva identificación de los componentes de una planta controvertida, *Cannabis sativa* L., mediante plataformas basadas en

cromatografía de gases–espectrometría de masas de tiempo de vuelo (GC–TOF/MS) y LC–QTOF MS/MS [6]. Estas investigaciones, que constituyen la Sección B de la tesis, abren nuevas líneas de trabajo en metabolómica vegetal.

-El exhaustivo estudio de una muestra apenas usada en clínica, el sudor, que ha consistido en: (1) el desarrollo de procedimientos para el muestreo y la preparación de la muestra — los primeros basados en estimulación de la sudoración mediante ejercicio moderado, mediante la forma convencional basada en estimulación química+eléctrica y por disolución de sudor seco utilizando soportes sólidos impregnados de los disolventes adecuados. (2) El análisis metabolómico de los dos primeros tipos de muestras y su comparación con sudor obtenido mediante el último tipo de muestreo [8,9]; todo lo cual forma parte de la Sección C. En todos los casos, el análisis no dirigido permitió establecer el perfil del tipo de muestra que debe obtenerse dependiendo de los metabolitos de interés (compuestos polares, no polares o de polaridad media), que se sometieron a análisis no dirigido para su identificación tentativa en todos los tipos de sudor muestreado.

La Sección C también recoge un estudio sobre muestreo de sudor después de ejercicio moderado, en el que se comparó su composición con la de sudor obtenido mediante muestreo pasivo utilizando diferentes procedimientos de inducción. Se ensayaron diferentes estrategias de preparación de muestra incluyendo estrategias de derivatización para obtener instantáneas representativas del metaboloma del sudor, especialmente de los metabolitos no polares, como corresponde al uso de una plataforma GC–MS [8].

Además, en la Sección C, se usaron como muestreadores de sudor seco diferentes soportes sólidos impregnados con diferentes disolventes y las composiciones de las muestras se compararon entre sí y también con la composición del sudor fresco. Todas las muestras se analizaron mediante dos plataformas, GC–MS y LC–MS/MS, y los resultados mostraron que el sudor seco es más adecuado para el análisis de los metabolitos de baja polaridad, mientras que el sudor fresco es más apropiado para compuestos polares [9].

La última de las investigaciones contenidas en la Sección C se refiere a aminoácidos existentes en sudor. Estos compuestos son metabolitos claves en la diagnosis y tratamiento de diversas enfermedades, como el cáncer, por lo que se determinan en diferentes biofluidos. La determinación cuantitativa de estos compuestos en sudor requirió la optimización de la preparación de la muestra que, dependiendo de la concentración, puede consistir en simple dilución o en microextracción centrífuga en fase sólida (c-SPμE) como

etapa previa a la inserción en un cromatógrafo de líquidos conectado a un espectrómetro de masas de triple cuadrupolo [7].

En los estudios de metabolómica clínica que se recogen en la Sección D se compararon diferentes estrategias de preparación de la muestra para la obtención del perfil de metabolitos en sudor con el uso de una plataforma GC–TOF/MS en modo de alta resolución [10]. La comparación mostró que una etapa de metoximación más sililación tras la desproteinización era la opción más adecuada para conocer la situación instantánea del metaboloma del sudor.

También la Sección D recoge el estudio de muestras de sudor procedentes de dos cohortes de pacientes de cáncer de pulmón, analizadas mediante LC–QTOF MS/MS para configurar paneles de biomarcadores con los que discriminar estos pacientes de fumadores como individuos con factor de riesgo [11]. El uso de una herramienta como PanelomiX permitió reducir los falsos negativos (95% de especificidad) y los falsos positivos (95% de sensibilidad) y la proposición de dos paneles de biomarcadores: uno, con 96.9% de especificidad y 83.8% de sensibilidad, compuesto por el monoglicérido MG(22:2), los ácidos mucónico, subérico y urocánico y una tetrahexosa; el otro con 81.2% de especificidad y el 97.3% de sensibilidad, compuesto por el monoglicérido MG(22:2), los ácidos mucónico, nonanodioico y urocánico, y una tetrahexosa.

3.Conclusión

La investigación realizada ha permitido extraer las siguientes conclusiones:

-La extensa búsqueda bibliográfica [1–3], ha permitido una evaluación crítica del estado actual de los métodos basados en USAE para obtener componentes de plantas/alimentos y de los controvertidos efectos de los US en las enzimas y en su acción biocatalítica, pero también ha proporcionado pautas para planificar investigación metabolómica de plantas, en la que es clave la asistencia de los US.

-La investigación experimental contenida en esta Memoria de Tesis se orientó al análisis metabolómico, tanto dirigido como global, en las áreas vegetal y animal. La diversidad de los estudios desarrollados incluye el efecto de la preparación de la muestra en la cobertura conseguida en la detección y el efecto favorable de los US en las reacciones enzimáticas. La variedad de los estudios realizados también abarca el efecto del muestreo y la preparación

de la muestra en la eficacia de la detección de componentes del sudor, así como el estudio de los cambios en los perfiles de los metabolitos en el sudor humano recogido después de estimulación mediante diferentes procedimientos.

Las conclusiones más destacables de la investigación en metabolómica en el área vegetal son:

- El método basado en USAEH para la obtención de la aglicona de la oleuropeína a partir de oleuropeína de extractos de hojas de olivo requiere menos de 20 min para la hidrólisis completa, lo que permite una comparación favorable con el método tradicional basado en incubación enzimática, que requiere 2 h [4].
- El fraccionamiento basado en extracción líquido–líquido secuencial con extractantes de diferente polaridad mejora drásticamente la cobertura de la identificación tentativa de metabolitos en los extractos enzimáticos acuosos del *A. bisporus* (123 metabolitos tentativamente identificados) [5].
- Se han identificado 169 compuestos (cannabinoides, terpenoides, lípidos, flavonoides, aminoácidos y ácidos orgánicos, entre los más destacables) en extractos polares y no polares, obtenidos con la asistencia de US, de 17 cultivos de *Cannabis sativa* L. Las plataformas GC–TOF/MS y LC–QTOF MS/MS maximizan la cobertura en la detección de componentes del cannabis, con lo que se ha consiguido identificar 46 compuestos en los extractos polares y 134 en los no polares [6].

En cuanto al efecto del muestreo y de la preparación de la muestra en la detección de metabolitos en sudor humano (Sección C), el uso de análisis dirigido y no dirigido permitió obtener las siguientes conclusiones:

- El sudor es una muestra excelente para el análisis cuantitativo del perfil de aminoácidos en estudios clínicos, lo que resulta de interés teniendo en cuenta el papel biomarcador de estos compuestos. La dilución+c-SPμE ha demostrado ser decisiva para disminuir el efecto matriz del sudor humano y hacer así posible la cuantificación de aminoácidos mediante LC–MS/MS [7].
- El sudor activo, con respecto al sudor pasivo, está enriquecido en algunas familias de compuestos claves, tales como ácidos grasos, alcoholes, carbohidratos y aminoácidos no proteinogénicos. La LLE con diclorometano y posterior metoximación más sililación es la mejor opción entre los protocolos ensayados para obtener mediante

GC–MS una instantánea representativa de los compuestos no polares del sudor activo [8].

- El muestreo de sudor seco es una alternativa para la estandarización de la recogida de sudor. El papel impregnado con etanol–PBS en proporción 1:1 (v/v) da lugar a los mejores resultados en términos de cobertura de la detección utilizando un análisis combinado mediante las plataformas GC–MS y LC–MS/MS. Se han detectado en sudor seco familias tales como carnitinas, esfingolípidos y N-acil-aminoácidos, que no aparecen o lo hacen a bajísima concentración en sudor fresco [9].

Los hallazgos más destacables en la mejora de la cobertura metabolómica del sudor para estudios clínicos han sido los siguientes:

- La optimización de una nueva plataforma basada en GC–MS para el análisis de sudor revela la presencia de metabolitos que pueden ser de interés para el estudio de diferentes patologías. Es destacable la capacidad de la plataforma para detectar azúcares, lípidos y ácidos carboxílicos, familias cuya presencia había resultado insignificante cuando se utilizaron plataformas basadas en RMN o en LC–MS [10].
- El diseño de dos paneles de cinco metabolitos que incluyen aminoácidos, ácidos dicarboxílicos de cadena corta, azúcares y algunos lípidos permite discriminar pacientes con cáncer de pulmón del grupo de control con factor de riesgo. Este nuevo modelo de predicción es más robusto que el obtenido previamente por nuestro grupo, ya que se construyó con dos tandas de muestras recogidas y analizadas con una diferencia de tiempo de dos años y por diferentes analistas [11].

4. Bibliografía

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Summary

1. Motivation of the thesis

The hypothesis that preceded the establishment of the objectives on which the research was planned were the existence of a large number of challenges that needed to be addressed in both plant and animal metabolomics. Therefore, the basic objective should encompass both metabolomics areas, and should be divided into plant and animal metabolomics. The three pillars on which the developed research was supported were the design of the appropriate analytical platform in each case, the supervision by senior researchers with experience in metabolomics, and the training+capability of the PhD student to accept and develop the planned challenges.

2. Content of the investigation

The performed research has involved:

-An in-depth study of the literature related to the planned work, complemented with a continuous updating during the development of the target studies that will keep the research in the front line. Especial attention have been paid to sample preparation and metabolomics, including a tool commonly used by the PhD student research team: ultrasound (US) used as auxiliary energy to favor analytical steps. Thus, US-assisted extraction (USAE) [1], US and metabolomics [2] and the controversial effect of US on enzymatic activity [3] gave place to three publications that constitute Section A of the thesis.

-The hydrolysis of oleuropein in olive leaf extracts accelerated by different hydrolases and by US action (USAEH) [4] (which opens the door to the complex enzymes–US world), and the use of a liquid chromatography–time-of-flight tandem mass spectrometry (LC–QTOF MS/MS) platform to obtain the data for tentative identification of 123 metabolites in extracts that had shown inhibition of the protease NS3 of hepatitis-C virus (HCV) [5] constitute key achievements. Another achievement was the in-depth identification of the components of a controversial plant, *Cannabis sativa* L., by gas chromatography–time-of-flight mass spectrometry (GC–TOF/MS) and LC–QTOF MS/MS platforms [6]. All these

investigations, which constitute Section B of the thesis, open new research lines in plant metabolomics.

-An in-depth study of an almost not used sample in clinical, sweat, has consisted of : (1) the development of sampling and sample preparation procedures —the first ones based on sweat stimulation of sweat by moderate exercise, by the conventional via based on chemical+electrical stimulation and on dissolution of dry sweat by using solid supports impregnated with appropriate solvents. (2) The metabolomics analysis of the first two types of samples and their comparison with sweat obtained by the last kind of sampling [8,9]; all of which is part of Section C. The untargeted analysis in all cases allowed setting the pattern of the type of sample to be obtained depending on the metabolites of interest (polar, no polar or medium polarity compounds), all which were subjected to untargeted analysis for tentative identification in all types of sampled sweat.

Section C also includes a study on sweat sampling after moderate exercise, in which the composition of this biofluid was compared with that of sweat obtained by passive sampling using different sweat induction procedures. Different sample preparation strategies, including derivatization strategies, were assayed to obtain a representative snapshot of sweat metabolome, mainly of no polar metabolites as corresponds to the use of a GC–MS platform [8]. Moreover, different solid supports impregnated with different solvents were used as dry sweat samplers and the composition of the collected samples was compared among them, and also with the composition of fresh sweat. All the samples were analyzed by a dual approach, GC–MS and LC–MS/MS, and the results showed that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds [9].

The last of the research contained in Section C refers to amino acids existing in sweat. These compounds are key metabolites in the diagnosis and treatment of several diseases such as cancer; therefore, they are determined in common biofluids. Quantitative determination of these compounds in sweat has required optimization of the sample preparation step which, depending on the concentration, can consist either on simple dilution or centrifugal microsolid-phase extraction (c-SPμE) prior to insertion into a liquid chromatograph connected to a triple quadrupole mass detector [7].

In the clinical metabolomics studies that constitute Section D, different sample preparation strategies were compared to obtain the profile of sweat metabolites by a GC–TOF/MS platform in high resolution mode [10]. The comparison showed that

methoxymation plus silylation after deproteination was the most suited option to obtain a representative snapshot of sweat metabolome.

Section D also contains the study of sweat samples, from two cohorts of lung-cancer patients, subjected to analysis by LC–QTOF MS/MS to configure biomarker panels to discriminate these patients from smokers as risk factor individuals [11]. The use of the PanelomiX tool allowed reducing false negatives (95% specificity) and false positives (95% sensitivity), and proposing two biomarker panels: one, with 96.9% specificity and 83.8% sensitivity, composed by monoglyceride MG(22:2), muconic, suberic and urocanic acids and a tetrahexose; and the other with 81.2% specificity and 97.3% sensitivity, and composed by monoglyceride MG(22:2), muconic, nonanodioic and urocanic acids, and a tetrahexose.

4. Conclusion

The performed research has allowed to extract the following conclusions:

-The in-depth bibliographic search [1–3] allows a critical evaluation of the state-of-the-art of USAE methods to obtain components from plants/foods, and the controversial effects of US on enzymes and their biocatalytic action, but also provides guidelines to plan the research on plants through metabolomics helped by US.

-The experimental research in this Thesis-Book was focused on the use of both targeted and untargeted metabolomics analysis for plant and clinical studies. The diversity of the developed studies includes the effect of sample preparation on detection coverage, and the favorable US effect on enzymatic reactions. The variety of the developed studies also covers the effect of both sampling and sample preparation on the efficient detection of sweat components, and the study of the changes in the metabolite profiles of human sweat collected after different sweat stimulation procedures.

The most outstanding conclusions from the research devoted to studying the metabolomics in the plant field are:

- The USAEH method to obtain oleuropein aglycon from oleuropein in olive leaf extracts requires less than 20 min for complete hydrolysis, which compares favorably with the traditional method based on enzymatic incubation, for which 2 h are necessary [4].

- Fractionation based on sequential liquid–liquid extraction with extractants of different polarity drastically improves the tentative identification coverage of metabolites in *A. bisporus* aqueous enzymatic extracts (123 tentatively identified metabolites) [5].
- A total of 169 compounds (cannabinoids, terpenoids, lipids, flavonoids, amino and organic acids, among others) have been identified in polar and no polar extracts obtained by US assistance from 17 cultivars of *Cannabis sativa* L. GC–TOF/MS and LC–QTOF MS/MS maximize the detection coverage of Cannabis components, so 46 compounds were identified in polar extracts and 134 compounds in no polar extracts [6].

Concerning the effect of sampling and sample preparation on the detection of metabolites from human sweat (Section C), untargeted and targeted analysis allowed reaching the following conclusions:

- Sweat is an excellent sample for quantitative profiling analysis of amino acids in clinical studies, which is of interest taking into account the biomarker role of these compounds. Dilution+c-SPμE has proved to be mandatory to decrease the matrix effect of human sweat thus making possible absolute quantitation of amino acids by LC–MS/MS [7].
- Active sweat, with respect to passive sweat, is enriched in some key families of compounds such as fatty acids, alcohols, carbohydrates and no proteinogenic amino acids. LLE with dichloromethane with subsequent methoxymation plus silylation is the best option among the tested protocols to obtain a representative snapshot of the no polar compounds from active sweat by GC–MS [8].
- Dry sweat sampling is an alternative to standardize sweat collection. Paper impregnated with 1:1 (v/v) ethanol–PBS provides the best results in terms of detection coverage by using a combined approach based on GC–MS and LC–MS/MS analysis. Particular families such as carnitines, sphingolipids and N-acyl-amino acids detected in dry sweat have not been reported or they do it at very low concentration in fresh sweat [9].

The most remarkable findings on enhancement of metabolomics coverage of sweat for clinical studies have been the following:

- The optimization of a new GC–MS platform for sweat analysis reveals the presence of metabolites that can be of interest for the study of different pathologies. It is worthy to remarking the ability of GC–MS to detect sugars, lipids and carboxylic acids, families that with previous platforms (NMR and LC–MS) seemed to have insignificant presence [10].
- The design of two panels of five metabolites including amino acids, short chain dicarboxylic acids, sugars and some lipids allow discriminating patients with lung cancer from a control group with risk factor. This new prediction model is more robust than that obtained previously by our group since it was built with two batches of samples collected and analyzed with a shift time of two years and by different analysts [11].

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Índice

Index

Objetivos

Hipótesis y objetivos de la investigación

La hipótesis que precedió al establecimiento de los objetivos sobre los que se planteó la investigación fue la existencia de un gran número de retos en metabolómica vegetal y animal de los que se requiere su abordaje.

Tras la presentación de la hipótesis, queda claro que el *objetivo básico* debe abarcar *ambas áreas de la metabolómica: la vegetal y la animal*. Los tres pilares en los que se soportó la investigación desarrollada fueron el diseño de la plataforma analítica apropiada para cada caso, la supervisión por investigadores con reconocida experiencia en metabolómica y la formación y capacidad de la doctoranda para aceptar y desarrollar los retos planteados; todos los cuales dieron lugar a los *objetivos específicos*, divididos en 4 secciones.

Sección A:

El *objetivo específico* de la Sección A debería ser el primer objetivo de cualquier investigación: llevar a cabo un estudio en profundidad de la bibliografía relacionada con el trabajo planteado, complementado con una actualización continua durante todo el desarrollo de la tesis para mantener la investigación en primera línea. De esta forma, además de adiestrarse en la literatura científica, el estudiante aprende a ser crítico con la información que maneja, de manera que puede contribuir a la formación de otros investigadores publicando su crítica y bien soportada información en revistas científicas accesibles. Los temas a los que la estudiante de doctorado ha prestado especial atención han estado relacionados con la preparación de la muestra y con la metabolómica, incluyendo una herramienta comúnmente utilizada por el grupo de investigación al que ella pertenece: los ultrasonidos (US) como energía auxiliar para favorecer etapas analíticas. De hecho, la extracción asistida por US es el tema del Capítulo I, los US y la metabolómica se discuten en el Capítulo II, mientras que el controvertido efecto de los US en la actividad enzimática constituye el Capítulo III.

Sección B:

Contribuir a la metabolómica vegetal abriendo nuevas líneas de investigación claves fue el *objetivo específico* de la Sección B. La hidrólisis de la oleuropeína en extractos de hojas de olivo acelerada por diferentes hidrolasas y por la acción de los US constituye el material del Capítulo IV, que abre la puerta al complejo mundo del binomio enzimas–US. El Capítulo V contiene el uso de una plataforma basada en cromatografía líquida y espectrometría de masas en tándem (LC–QTOF MS/MS) con la que obtener los datos para la identificación tentativa de 123 metabolitos, 55 de los cuales fueron identificados por primera vez en extractos enzimáticos acuosos de la seta comestible *Agaricus bisporus*. Los extractos habían producido la inhibición de la proteasa NS3 del virus de la hepatitis-C (HCV); por tanto, la instantánea que proporciona este estudio es muy prometedora para los pacientes con HCV. En la misma línea, la investigación del Capítulo VI puede ser un avance clave en medicina, ya que está dedicado a la identificación amplia de los componentes de una planta controvertida: *Cannabis sativa* L. Se identificaron un total de 169 compuestos en extractos polares y no polares en 17 cultivos de *Cannabis sativa* L. utilizando los datos obtenidos mediante plataformas basadas en cromatografía de gases–espectrometría de masas de tiempo de vuelo (GC–TOF/MS) y LC–QTOF MS/MS. La existencia de patrones comerciales de cannabinoides y terpenoides posibilitó confirmar la identificación de un buen número de ellos. Se compararon los contenidos relativos de terpenoides y cannabinoides de los mismos cultivares procedentes de invernadero y de campo: las diferencias en composición entre ambos tipos de cultivos abren una puerta a un cultivo más racional de esta planta en función del uso al que se destine.

Secciones C y D:

El *objetivo específico* común de las Secciones C y D fue un estudio en profundidad de una muestra apenas usada en clínica: el sudor. Por tanto, se abordaron procedimientos para el muestreo y la preparación de la muestra. Los procedimientos de muestreo se basaron en estimulación de la sudoración mediante ejercicio moderado, mediante la forma convencional basada en estimulación química+eléctrica y por disolución de sudor seco utilizando soportes sólidos impregnados de los disolventes adecuados. El análisis metabolómico de los dos primeros tipos de muestras y su comparación con sudor obtenido mediante el último tipo de muestreo constituyen los Capítulos VIII y IX, respectivamente.

En todos los casos, el análisis no dirigido permitió establecer el perfil del tipo de muestra que debe obtenerse dependiendo de los metabolitos de interés (compuestos polares, no polares o de polaridad media), todos los cuales se sometieron a análisis no dirigido para su identificación tentativa en todos los tipos de sudor muestreado.

El muestreo de sudor después de ejercicio moderado proporcionó muestras de diferentes partes del cuerpo y su composición se comparó con la de sudor obtenido mediante muestreo pasivo utilizando diferentes procedimientos de inducción. Se ensayaron diferentes estrategias de derivatización para obtener instantáneas representativas del metaboloma del sudor, especialmente de los metabolitos no polares, como corresponde al uso de una plataforma GC–MS. El Capítulo VIII refleja los resultados de esta investigación.

El muestreo de sudor seco constituye una novedad, por lo que se utilizaron como muestreadores diferentes soportes sólidos impregnados con diferentes disolventes y las composiciones de las muestras se compararon entre sí y también con la composición de sudor fresco. Todas las muestras se analizaron mediante dos plataformas, GC–MS y LC–MS/MS, y los resultados permitieron concluir que el sudor seco es más adecuado para el análisis de los metabolitos de baja polaridad, mientras que el sudor fresco es más apropiado para compuestos polares, tal como se recoge en el Capítulo IX.

La plataforma GC–TOF/MS en modo de alta resolución soportó la comparación de diferentes estrategias de preparación de la muestra para la obtención del perfil de metabolitos en sudor, entre ellas la metoximación más sililación después de la desproteinización fue la opción más adecuada para obtener una instantánea del metaboloma del sudor. La identificación tentativa de 66 metabolitos (la mayoría de ellos implicados en rutas bioquímicas claves) y las excelentes características del método desarrollado abren nuevas posibilidades al uso del sudor como fuente de metabolitos biomarcadores de desórdenes específicos. Todos estos logros constituyen el Capítulo X.

Muestras de sudor procedentes de dos cohortes de pacientes de cáncer de pulmón se sometieron a análisis mediante LC–QTOF MS/MS para configurar paneles de biomarcadores con los que discriminar estos pacientes de fumadores como individuos con factor de riesgo. El uso de una herramienta como el PanelomiX permitió reducir los falsos negativos (95% de especificidad) y los falsos positivos (95% de sensibilidad), y la proposición de dos paneles de biomarcadores: uno, con 96.9% de especificidad y 83.8% de

sensibilidad, compuesto por el monoglicérido MG(22:2), los ácidos mucónico, subérico y urocánico y una tetrahexosa; y el otro con 81.2% de especificidad y el 97.3% de sensibilidad, y compuesto por el monoglicérido MG(22:2), los ácidos mucónico, nonanodioico y urocánico, y una tetrahexosa. El modelo de predicción así obtenido constituye el Capítulo XI.

Los aminoácidos son el *objetivo específico* abordado en el Capítulo VII. Estos compuestos son metabolitos claves en la diagnosis y tratamiento de diversas enfermedades, como el cáncer; por tanto, se determinan en diferentes biofluidos. La determinación cuantitativa de estos compuestos en sudor requirió la optimización de la preparación de la muestra, que, dependiendo de la concentración, puede consistir en simple dilución o en microextracción centrífuga en fase sólida como etapa previa a la inserción en un cromatógrafo de líquidos conectado a un espectrómetro de masas de triple cuadrupolo, tal como se detalla en el Capítulo VII.

El *objetivo final* de toda Tesis Doctoral debe ser siempre la formación del futuro doctor o doctora. En el presente caso, su formación ha incluido también los cursos obligatorios de un máster en química. En paralelo con las tareas antes comentadas y la investigación recogida en la parte principal de esta Memoria de Tesis, la formación de la doctoranda se ha ampliado con el desarrollo de otras actividades, que se resumen a continuación como anexos.

Anexo I: Artículos de revisión sobre temas relacionados con la Tesis, tales como emulsificación–extracción asistida por US, extracción asistida por US y derivatización *in situ* e “in medium virtus” evaluación de la química analítica verde.

Anexo II: Capítulo de libro sobre un tema relacionado con la Tesis, como es el uso de las microondas en el campo de las ómicas.

Anexo III: Experto en Docencia Universitaria, con título propio de la Universidad de Córdoba.

Anexo IV: Artículo de investigación derivado de un Proyecto del Plan de Innovación y Buenas Prácticas Docentes (desarrollo de un método para la evaluación de la adquisición de competencias y su comparación con el sistema tradicional de evaluación en asignaturas

del Grado de Química), que fue el resultado de la colaboración con miembros del Departamento de Química Física y Termodinámica Aplicada.

Anexo V: Codirección del Trabajo Fin de Máster (TFM) de Francisco Martín Loro (La orina como muestra clínica para la búsqueda de biomarcadores metabolómicos de cáncer de próstata sustitutivos del PSA), desarrollado en la UCO durante el curso académico 2017–18.

Anexo VI: Codirección de los Trabajos Fin de Grado (TFG) de Ismael Ruiz González (Caracterización de extractos fenólicos de hoja de olivo para la preparación de concentrados purificados con interés nutricional), de Eva Natalia Cárdenas Soria (Comparación del espectrómetro de diodos en fila y el de masas conectados a un cromatógrafo de líquidos para la cuantificación en orina de metabolitos biomarcadores de cáncer de próstata) y de Elvira Cruces Vera (Comparación del perfil metabólico de los componentes del sudor en niños, adultos y ancianos), desarrollados en la UCO durante los cursos académicos 2018–19, 2017–18 and 2016–17, respectivamente.

Anexo VII: Comunicaciones orales y en carteles en congresos y reuniones nacionales de química analítica.

Anexo VIII: Comunicaciones orales y en carteles en congresos y reuniones de investigadores jóvenes.

Anexo IX: Comunicaciones orales en congresos de medicina.

Objectives

Hypothesis and objectives of the research

The hypothesis that preceded the establishment of the objectives on which the research was planned were the existence of a large number of challenges that needed to be addressed in both plant and animal metabolomics.

After presentation of the hypothesis, it was clear that the *basic objective* should encompass both metabolomics areas, and *should be divided into plant and animal metabolomics*. The three pillars on which the developed research was supported were the design of the appropriate analytical platform in each case, the supervision by senior researchers with experience in metabolomics, and the training+capability of the PhD student to accept and develop the planned challenges; all them giving place to the *specific objectives* divided into 4 sections.

Section A:

The *specific objective* of Section A should always be the first objective of any research: to carry out an in depth study of the literature related to the planned work, complemented with a continuous updating during the development of the target studies that will keep the research in the front line. In this way, in addition to training in literature, the PhD student learns to be critical with the information she handles in such a way that she can contribute to the training of other researchers by publishing her well supported and critical information in accessible journals. The subjects to which the PhD student has paid especial attention have been related to sample preparation and metabolomics, including a tool commonly used by the research team to which she belongs: ultrasound (US) as auxiliary energy to favor analytical steps. Thus, US-assisted extraction is the matter of Chapter I, US and metabolomics are discussed in Chapter II, while the controversial effect of US on enzymatic activity constitutes Chapter III.

Section B:

To contribute to plant metabolomics by opening new key research lines was the *specific objective* of Section B. The hydrolysis of oleuropein in olive leaf extracts accelerated

by different hydrolases and US action constitutes the matter of Chapter IV, which opens the door to the complex enzymes–US world. Chapter V contains the use of a liquid chromatography–time-of-flight tandem mass spectrometry (LC–QTOF MS/MS) platform to obtain the data for tentative identification of 123 metabolites, 55 of which were identified for the first time in aqueous enzymatic extracts of the common edible mushroom *Agaricus bisporus*. The extracts had shown inhibition of the protease NS3 of hepatitis-C virus (HCV); therefore, the representative snapshot of the composition of the target extracts thus obtained is very promising for HCV patients. In this line, the research in Chapter VI may constitute a key advance in medicine as it is devoted to in-depth identification of components of a controversial plant: *Cannabis sativa* L. A total of 169 compounds were identified in polar and no polar extracts from 17 cultivars of *Cannabis sativa* L. using the data obtained by gas chromatography–time-of-flight/mass spectrometry (GC–TOF/MS) and LC–QTOF MS/MS platforms. A number of commercial standards of cannabinoids, and terpenoids allowed confirming their identification. Relative contents of terpenoids and cannabinoids in the same cultivars grown in greenhouse and in field were compared: the compositional differences between both types of grown conditions open a door for a more rational cultivation of this plant as a function of its use.

Sections C and D:

Common *specific objective* of Sections C and D was an in-depth study of an almost no used sample: sweat. Therefore, sampling and preparation procedures for this clinical sample were afforded. Sampling procedures were based on sweat stimulation by moderate exercise, by the conventional via based on chemical+electrical stimulation and on dissolution of dry sweat by using solid supports impregnated by appropriate solvents. The metabolomics analysis of the first two types of samples and their comparison with sweat obtained by the last kind of sampling constitute Chapters VIII and IX, respectively. The untargeted analysis in all cases allowed setting the pattern of the type of sample to be obtained depending on the metabolites of interest (polar, no polar or medium polarity compounds), all which were subjected to untargeted analysis for tentative identification in all types of sampled sweat.

Sweat sampling after moderate exercise provided samples from different parts of the body, the composition of which was compared with that of sweat obtained by passive

sampling and using different sweat induction procedures. Different sample preparation strategies including derivatization were assayed to obtain a representative snapshot of sweat metabolome, mainly of no polar metabolites as corresponds to the use of a GC–MS platform. Chapter VIII contains this research.

Sampling dry sweat constitutes a novelty; therefore, different solid supports impregnated with different solvents were used as samplers and the composition of the collected samples was compared among them, and also with the composition of fresh sweat. All the samples were analyzed by a dual approach, GC–MS and LC–MS/MS, and the results allowed concluding that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds, as shows Chapter IX.

The GC–TOF/MS platform in high resolution mode supported the comparison of different sample preparation strategies on the profile of sweat metabolites, among which methyoxiamination plus silylation after deproteination was the most suited option to obtain a representative snapshot of sweat metabolome. The tentative identification of 66 metabolites (most of them involved in key biochemical pathways), and the excellent characteristics of the developed method open new possibilities to the use of sweat as a source of metabolite biomarkers of specific disorders. All these achievements constitute Chapter X.

Sweat samples from two cohorts of lung-cancer patients were subjected to analysis by LC–QTOF MS/MS looking for proposing biomarkers panels to discriminate these patients from smokers as risk factor individuals. The use of the PanelomiX tool allowed reducing false negatives (95% specificity) and false positives (95% sensitivity), and proposing two biomarkers panels: one, with 96.9% specificity and 83.8% sensitivity, composed by monoglyceride MG(22:2), muconic, suberic and urocanic acids, and a tetrahexose; and the other with 81.2% specificity and 97.3% sensitivity, and composed by the monoglyceride MG(22:2), muconic, nonanedioic and urocanic acids, and a tetrahexose. The prediction model thus obtained constitutes Chapter XI.

Amino acids are the *specific objective* afforded in Chapter VII. They are key metabolites in the diagnosis and treatment of several diseases as cancer; therefore, they are determined in common biofluids. Quantitative determination of these compounds in sweat have required optimization of the sample preparation step that can consist either of simple dilution or centrifugal microsolid-phase extraction, depending on the concentration of the

metabolites, prior to insertion into a liquid chromatograph connected to a triple quad mass detector, as shown in Chapter VII.

The *final objective* of a Doctoral Thesis should be always the formation of the future PhD. In the present case her formation has also included the mandatory courses of a master on Chemistry. In parallel to the above-mentioned tasks and the research in the main part of this Thesis-Book, the training of the PhD student has been improved by development of other activities below summarized as annexes:

Annex I: Review articles on subjects related to the Thesis, as are US-assisted emulsification–extraction, US-assisted extraction and *in situ* derivatization, and the "in medium virtus" assessment of green analytical chemistry.

Annex II: Book chapter on a subject related to the Thesis, as is microwaves in the omics field.

Annex III: Expert in University Teaching, graduating from the University of Córdoba.

Annex IV: Research article from a Project of the Innovation Plan and Good Teaching Practices (development of a method for the evaluation of competences acquisition and its comparison with the traditional evaluation system in subjects of Chemistry degree), that resulted from the collaboration with members of the Department of Physical Chemistry and Applied Thermodynamics.

Annex V: Co-supervision of the Master´s Thesis of Francisco Martín Loro (La orina como muestra clínica para la búsqueda de biomarcadores metabolómicos de cáncer de próstata sustitutivos del PSA), developed in the UCO during the 2017–18 academic year.

Annex VI: Co-supervision of the Final Degree Projects (TFG) of Ismael Ruiz Gónzalez (Caracterización de extractos fenólicos de hoja de olivo para la preparación de concentrados purificados con interés nutricional), Eva Natalia Cárdenas Soria (Comparación del espectrómetro de diodos en fila y el de masas conectados a un cromatógrafo de líquidos para la cuantificación en orina de metabolitos biomarcadores de cáncer de próstata), and Elvira Cruces Vera (Comparación del perfil metabólico de los componentes del sudor en niños, adultos y ancianos), developed in the UCO during the 2018–19, 2017–18 and 2016–17 academic years, respectively.

Annex VII: Oral and poster communications in national analytical chemistry meetings.

Annex VIII. Oral and poster communications in young investigators meetings.

Annex IX. Oral communications in medicine meetings.

Introducción

Introduction

INTRODUCTION

An overview of the knowledge acquired by the PhD student on analytical concepts and tools training is intended to be shown in this introduction by encompassing: (i) the basic aspects on metabolomics, on which the research presented in this Thesis-Book has been supported. (ii) The sampling, preliminary and sample preparation steps needed before both plant and clinical metabolomics analyses. (iii) The analytical platforms solidly supported on mass spectrometry (MS) equipment managed by the PhD student, as support for targeted and untargeted analyses. (iv) The chemometric approaches used for treatment of the multiple data provided by the analytical platforms she developed. Some of the existing gaps in plant and clinical metabolomics have been successfully afforded by the PhD student helped by the knowledge and training she acquired, as shows the present Thesis-Book. All the achievements included in it are supported solidly on the previous experience and research developed by the group of which she is a part of, and on a continuous and appropriate supervision.

1. Metabolomics

The "omics of small molecules", as is also named the youngest of the primary omics, constitutes, together with those that preceded it —*viz.*, genomics, transcriptomics and proteomics—, the solid support of the present systems biology. Information/discussion on metabolomics, as the omics on which the research developed by the PhD student, is developed below.

1.1. Definitions of the discipline

A number of definitions of metabolomics have appeared in the literature since it was considered as a new omics. The first time the term metabolome appeared in the literature was 1998, once Oliver *et al.* measured the change in the relative concentrations of metabolites as a consequence of overexpression or deletion of a gene [1]. Therefore, the metabolome refers to the complete set of small compounds (such as metabolic intermediates, hormones and other signaling molecules and secondary metabolites) found in a living organism, either animal or plant. It represents the ultimate phenotype of cells,

deduced from the perturbation of gene expression and the modulation of protein function, as well as environmental cues.

One year later that the term metabolome was defined, the term metabonomics was used by Nicholson *et al.* [2], who defined it as "quantitative measurement of the dynamic multiparametric metabolic response of living systems to physiopathological stimuli or genetic modifications". Then, the term metabolomics was first defined by Fiehn in 2001, as "an overall and exhaustive analysis by which all the metabolites in a biological system are identified and quantified" [3]. So, both terms, "metabolomics" and "metabonomics", were generated almost at the same time to describe the same discipline, but in the plant and clinical/pharmaceutical fields, respectively [4,5]. Independently of the differences in the name of this omics, a question must be posed from the definitions: may metabolomics be considered "a quantitative measurement" or "an analysis", exhaustive or not? A more generic and neutral term "discipline" —or even "science"— seems to be more appropriate to support the definition of metabolomics. Therefore, the current definition of metabolomics as "the discipline that provides comprehensive and systematic information on temporal changes in the profiles of metabolite levels in biofluids and tissues, which can arise from control by the host genome, extended genomes and effect of other environmental or promoted factors" is adjusted better to the present functions of metabolomics. The use of metabolomics in both animal and plant fields is practically accepted at present.

In the meantime, a number of more or less acceptable definitions of metabolomics had been proposed. The definition of metabolomics as "the untargeted measurement of the metabolome, which is composed of the complement of small molecules detected in a biological sample" [6] deletes from it targeted analysis, which is considered erroneously as simple multianalysis. This definition involving only untargeted measurements contrasts with that established by the Metabolomics Society as "a newly emerging field of omics research concerned with the comprehensive characterization of the small molecule metabolites in biological systems" [7]. In this case, metabolomics is not identified as analysis or measurement but related to characterization of small molecules that provide an overview of both the global biochemical processes and metabolic status related to a biological or a cellular system. The involvement of "metabolites characterization" (in contrast to genome sequencing) makes glimpse the present practical impossibility of comprehensive metabolomics owing to: (i) the enormous chemical diversity of a typical metabolome, which requires multiple general approaches for extraction, fractionation and

analysis to accommodate variation in solubility, reactivity and other physico-chemical properties [8]; (ii) the dynamic range of metabolite concentrations, which can also be huge requiring efficient separation strategies to avoid that minor components are not completely lost among those that are more abundant; (iii) the spatial distribution, which includes specific organs, cell types, sub cellular domains, extracellular spaces and occasionally extended beyond the organism to compounds excreted into the environment [9]; (iv) the temporal distribution, similarly large with variations ranging across an organism's lifespan, seasonal and circadian rhythms and faster chemical movements and oscillations [10]; and (v) the unlike proteomics and transcriptional studies, genomic information cannot be used as a constraint for the identification of molecular species.

As a part of the primary omics, metabolomics has also been defined as "an essential component of systems biology, and metabolism studies abound in fields ranging from agriculture to the study of human diseases" [11] —where metabonomics had been forgotten in dealing with clinical metabolomics studies. One of the most recent definitions of metabolomics establishes that "metabolomics is mainly based on the quantitative measurement of dynamic metabolic changes of living systems in response to genetic modifications or physiological stimuli, including nutrients and drugs [12]. By the global study of low molecular weight metabolites (<1500 Da) in biofluids (plasma/serum and urine) and tissues, metabolomics assures the characterization of an individual metabolic phenotype".

In short, metabolomics provides information that would conduct to the whole metabolome, which is the quantitative complement of low-molecular weight metabolites existing in a biological cell, organism or fluid under a set of physiological conditions or perturbations (*e.g.*, pathological states, genetic variations or responses to external stimuli) [13]. Nevertheless, when the state, variation or response to an external stimulus can be monitored by one metabolite or a family of metabolites, it is also a metabolomics study: a targeted metabolomics study.

1.2. Relationship of metabolomics with other great omics

Omics studies involve measurement of large numbers of parameters —typically genes (genomics), mRNA (transcriptomics), proteins (proteomics), or metabolites (metabolomics)— in dealing with individual *primary omics*.

Overall and wide information on the dynamics and structure or functions of the cells and organisms to understand biology at the system level is obtained by the joint application of two or more omics. The combination of the data from the different omics constitutes systems biology, which allows a better comprehension of the functioning of a biological system. Presently, systems biology is better known as integrative omics [14].

The fundamental differences between the traditional central dogma of molecular biology, and systems biology (integrative omics) result from the flow of biological information they provide, as visualized in the comparison of Fig. 1 with Fig. 2. In the former the flow of information is unidirectional —from genes of a cell or organism (*viz.*, DNA) to their transcription at mRNA and their translation to proteins, ending with the catalysis of metabolic reactions and being finally revealed in the phenotype.

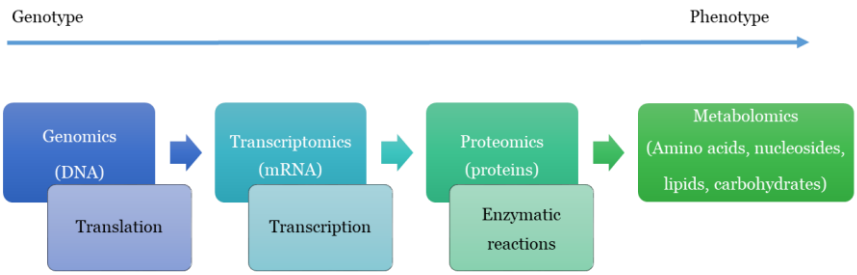

Fig. 1. Traditional central dogma of molecular biology.

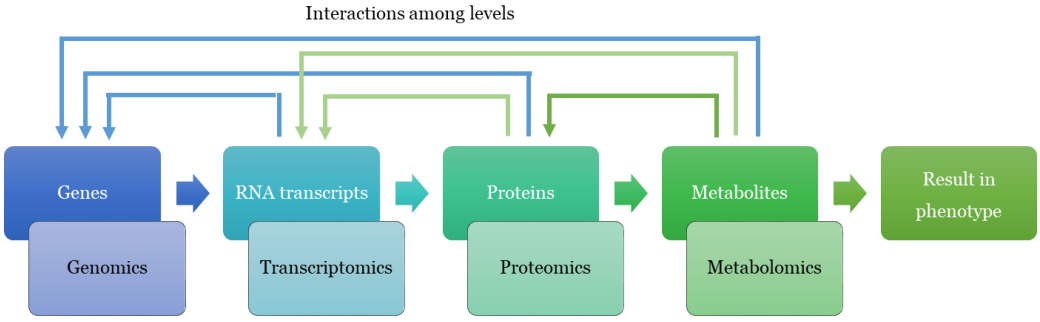

Fig. 2. General scheme of systems biology (omics integration).

On the contrary, omics integration allows no only the flow of biological information to go from genes to transcripts to proteins to metabolites, but more importantly, this flow can also establish bottom-up positive and negative feedback control. Thus, information on proteins, transcripts and/or genes by feedback loops (Fig. 2) can be obtained, either overall information or up-stream information from an omics located lower in the flow of the traditional central dogma of molecular biology.

1.3. Metabolomics subdisciplines

The vast field of metabolomics makes mandatory division into concrete areas with limited content. Among them, distinction must be made among primary metabolomics subdisciplines (*e.g.*, lipidomics, nutrimetabolomics or nutritional metabolomics, xenometabolomics), and other minor subdisciplines such as pharmacometabolomics, environmental metabolomics, cardiometabolomics, secretomics, cosmetobolomics, microbial metabolomics and many others that have emerged and will continue emerging as a result of the importance acquired by application of metabolomics in new sectors.

Among primary metabolomics subdisciplines, lipidomics is the most relevant, even considered by some authors as a discipline independent of metabolomics (despite it is constituted by metabolites). Lipidomics, as other metabolomics subdisciplines, can be defined by a simple criterion as "discipline for complete characterization of all lipids in a particular type of cell", or by a wider and more complex criterion as "discipline to obtain overall information on all lipids in a biological system with respect to cellular signaling, membrane architecture, transcriptional and translational modulation, cell–cell and cell–protein interactions, and response to environmental changes with time", both definitions excluding targeted lipidomics, despite this strategy is each time more accepted by metabolomics researchers.

Other classification of metabolomics subdisciplines is based on the covered scope, which allows division into animal metabolomics (including clinical metabolomics) and plant metabolomics. These two areas have been subjects of the research developed by the PhD student, the results of which constitutes the main part of this PhD-Book.

1.4. Metabolomics strategies

Metabolomics analysis encompasses different strategies the nature of which depends on the objective of the study and previous knowledge of the biological problem [15]. The two more common and more widely used strategies in metabolomics are untargeted and targeted analyses.

1.4.1. Untargeted analysis. This is the metabolomics strategy par excellence as it affords primarily the qualitative or semiquantitative analysis of the largest possible number of metabolites from a diversity of chemical and biological classes contained in a biological specimen [16]. Detection of this wide range of metabolites can be done through a single analytical platform or a combination of complementary platforms, mainly based on nuclear magnetic resonance (NMR) or mass spectrometry (MS), coupled to gas chromatography (GC), liquid chromatography (LC) or, less commonly, capillary electrophoresis (CE). In these studies, the relative concentrations of the analyzed metabolites are generally calculated and their variations between two or more system situations are studied. In untargeted approaches, the sample preparation step keeps as simple as possible to obtain the widest metabolite coverage. No selective sample pretreatments such as protein precipitation for plasma/serum or simple dilution for urine samples (referred to as dilute-and-shoot) are generally used [17,18]. Besides, the data set obtained by applying these strategies is very extensive, so it requires treatments with advanced chemometric approaches for conversion into manageable signals and, finally, interpretable results. The signals require annotation using either available experimental libraries, structural elucidation by *in silico* fragmentation tools or experimental identification. Thus, this strategy allows identification of new metabolites that are involved in metabolic pathways and, therefore, the knowledge of them. Untargeted analysis was the most used strategy for the research developed and collected in this Thesis-Book. It was selected to obtain as much information as possible from the compounds present in plant extracts (Chapters V and VI), and in a clinical sample such as sweat with an almost unknown composition (Chapters VIII-to-X).

1.4.2. Targeted analysis. This strategy is either a quantitative approach in which concentrations are determined or a semiquantitative approach in which relative signals are recorded, always referred to few metabolites and/or substrates of metabolic reactions that might be associated to common chemical classes or linked to selected metabolic pathways [19]. The fact that the number of metabolites involved in this strategy is limited and their

nature known, led to researchers to consider studies developed using targeted analysis as not pertaining to metabolomics. Nevertheless, this strategy is always applied after untargeted analysis that allows knowing the metabolites through which the changes in a given organism or in a biological process can be studied. The previous knowledge of the metabolites involved in the given process allows selection of the most appropriate analytical technique and a selective sample preparation depending on the nature and abundance of the target metabolites, enabling a sufficient sample cleanup and preconcentration, if required. A mass spectrometer is the detector of choice —particularly the triple quadrupole (QqQ) approach— in targeted metabolomics because of its sensitivity. These strategy and instrument were selected to develop the studies in Chapters IV and VII for the determination of oleuropeine and amino acids, respectively.

Other strategies less used are as follows:

1.4.3. Metabolomics fingerprinting. This strategy was defined by Fiehn as a high throughput, a fast methodology for analysis of biological samples that provides fingerprints for sample classification and screening [20]. Fingerprinting is not focused on particular metabolite(s); therefore, it allows the discovery of novel metabolic pathways disturbed by the disease or preset stimulus. This approach is frequently used prior to a more comprehensive strategy (metabolomics profiling) to find discrimination patterns among groups of samples.

1.4.4. Metabolomics footprinting. This was the term proposed in 2005 by Kell *et al.* to refer to the study of metabolites in extracellular fluids, also known as exometabolome or secretome [21]. The purpose of this strategy is mainly to obtain information for classification of groups of samples or for screening.

Both finger- and footprinting strategies are commonly used in cell culture studies and their join application in this field is of clear interest as they are highly complementary when used in combination.

The detection techniques involved in finger- and footpringing also depend on the chosen strategy. For example, to obtain a metabolomic fingerprint the detection technique should allow direct and rapid analysis of the sample. NMR, MS (depending on the complexity of the sample) and, to a lesser extent, infrared and Raman spectroscopies are mainly used in this context. A separation technique is generally applied in both targeted

and untargeted analysis prior to individual detection of the metabolites for their quantitation or identification, being GC or LC the most used in this area.

In dealing with metabolomics coverage, it is always a compromise between quality of data and throughput of analysis. Thus, while global metabolic profiling and metabolic fingerprinting are tools for large metabolite coverage, the quality of the data is lower than in targeted profiling, where the method developed is exclusively optimized for one metabolite or few metabolites [22]. However, a large volume of data is required for high coverage, which is clearly opposed to high-throughput analysis.

1.5. Current status of metabolomics and some of its pending aspects

A reflection on the present and future perspectives of metabolomics is convenient to be expressed as a final subsection of this section devoted to the youngest of the primary omics.

Of the few thousand metabolites we can currently assign either unambiguously or with a high level of confidence, the precise *in vivo* function of many of them is unknown, and even their spatial abundance is not always clearly understood. Nevertheless, it must be pointed out that the combination of standard metabolomics approaches with studies of wide natural variance and the use of either genome-editing techniques or combinatorial transformation potentially offer powerful tools by which the function of an individual metabolite can be assigned.

An aspect that was inconceivable a decade ago is that metabolomics has become an essential tool in the elucidation of functional annotation of genes associated with metabolism. For this purpose, three major approaches have been used: (i) direct testing of candidate genes via the analysis of knock-out mutants; (ii) quantitative trait loci mapping of the genes determining the abundance of specific metabolites; and more recently (iii) genome-wide association studies of metabolite abundance.

In addition to the research that details how metabolomics has been used to define the way in which the general metabolic landscape changes in response to genetic or environmental perturbation [23], an approach to understand metabolite function has recently emerged probing metabolite–protein interactions [24,25]. It is commonly

performed by the co-elution of metabolites and proteins following the separation of the proteins.

A couple of emergent strategies warrant also a mention here: the use of isotope labeling and the development of metabolite imaging techniques. The first approach has already been demonstrated to be a powerful way of improving the annotation of plant metabolites [26], as well as providing powerful information concerning both the elucidation of pathway structures [27] and gene function annotations [28]. Metabolite imaging techniques meanwhile provide high or even ultrahigh spatial resolution of metabolite abundances [29]; however, to date their coverage is currently relatively limited.

A vital question is why has the specific function of most plant metabolites not yet been characterized? One problem is that their biological properties are largely described for a class of compounds rather than for individual metabolites. Furthermore, the promiscuity of many enzymes of specialized metabolism (which produce the majority of plant metabolites), alongside the general intricacy of most metabolic networks, renders it difficult to modify the content of a single metabolite without affecting the others. These features of metabolism hence make very difficult to directly assess metabolite function via conventional reverse-genetics strategies. For this purpose, more sophisticated approaches, in which the kinetic properties of the respective enzymes experience alteration, are likely to be required.

Some of the key questions in metabolomics are as follows: (i) how best do we further advance the coverage of the metabolome? (ii) Despite a growing appreciation of the importance of interactions between metabolites and other molecular entities, by contrast to other systems, very few metabolite receptors have been reported in plants yet. (iii) What is the best strategy to dissect the function of individual metabolites?

2. Basic differences between plant and animal metabolomics

2.1. Primary and secondary metabolites

Before starting with the differences between the metabolomics of the two kingdoms, attention must be paid to the difference between primary and secondary metabolites, the compilation of which constitutes the metabolome: the final recipient of

genetic information. Whereas genes and proteins are mostly involved in unfolding information needed for actualization of cellular functional processes, metabolite patterns reveal the actual dynamic cellular environment. In general, metabolites are intermediate or final products from metabolism, divided into primary and secondary metabolites, which constitute the products of molecular pathways disturbed by other omics; therefore, the metabolome defines the individual phenotype, by contrast with the transcriptome or the proteome. Primary or essential metabolites (*e.g.*, carbohydrates, vitamins, amino acids, nucleosides, organic acids) sustain the organism life via normal metabolic processes such as respiration or photosynthesis, and are involved directly in the normal growth, development and reproduction of the given organism. Secondary or no essential metabolites (*e.g.*, phenols, alkaloids, steroids, toxins*)* are no directly involved in growth, development and reproduction, but they have key ecological functions (*e.g.*, to defend against predators, parasites or diseases, competition between species or to facilitate the processes of reproduction by colors and/or smells). The interaction of both types of metabolites (both in animals and plants) is probably one of the most active regulatory circuits for balancing environmental pressures on the given system, either biotic or abiotic.

2.2. Definition of plant and animal (or clinical) metabolomics

Plant metabolomics is the science or discipline devoted to the study of the changes produced in a given plant population when subjected to a change caused by evolution, stress, genetic modification, etc. This discipline provides information on nutrients and vitamins, and genetic engineering of plants to produce more nutrients and vitamins in the edible portions of the plant is a key area of plant metabolomics research [30]. In fact, the information learned from metabolomics studies in plants has the potential for great impact in improving plant response to stress, agricultural efficiency, and food quality.

Many of the secondary metabolites are also referred to as plant natural products and have historically been a key source of lead molecules in drug discovery due to their action on many pharmacological targets [31]. Depending on the variety of cultivars (different accession), metabolites from the same plant can differ considerably, even if they are grown under the same conditions. Their phenotypic plasticity enables plants to withstand environmental dynamism, within both short and long time scales, and is governed by genes that not only determine the character of an organism, but also the degree

of responsiveness of that character to environmental stimuli [32]. Phenotypic plasticity is measured by the plant ability to change its growth pattern (morphological traits) and function (metabolic traits) as response to changes of environmental pressures through to activation or up-regulation of highly specialized metabolic pathways [33].

Animal metabolomics is the discipline that studies the metabolic reaction to medication, environment and/or diseases. Clinical metabolomics is aimed at evaluating the health status of an individual as well as the risk of disease by any of the different metabolic analysis of biofluids or tissues, which are influenced by their genetic, epigenetic, environmental exposures, diet and behavior.

The metabolome of an animal organism changes in response to stimuli and environmental conditions. This does not happen with its genome or its transcriptome, which only change when a genetic mutation occurs, being practically constant throughout its life. Diseases affect metabolism and, as a result, produce changes of sufficient duration to be detected by measurements of the metabolites [34]. Examples of this ability include schizophrenia, depression, Alzheimer's, cardiovascular disease, hypertension, type 2 diabetes, cancer or Huntington's disease [35]. These diseases are characterized by changes (decrease–increase) in the concentration of dozens of metabolites with respect to homeostasis. The analysis of the metabolite profile can provide information of these pathologies, very useful to combat them.

2.3. Intrinsic differences between animal and plant metabolomics

Intrinsic, key metabolomics differences between the plant and animal kingdoms must be clearly discussed as they establish the characteristics of the steps involved in the analytical process used to obtain the required information from the given system. The key differences between them are as follows:

2.3.1. Sessility/motility. The primary and biggest difference between animals and plants is the motility of most of the former and sessility of plants, which clearly influence their metabolism. Because plants are sessile organisms, they cannot escape from changing environmental conditions and/or plant–attacker interactions that adversely affect their growth and development (Fig. 3). Therefore, plant survival mostly depends on the initiation of complex adaptive responses that involve stress sensing, signal transduction and the activation of a number of stress-related genes and metabolites [36]. Central metabolism is

involved in the regulation of the various developmental processes that allow plants to survive such environmental threats, and the measurement of known primary metabolites (*e.g.*, carbohydrates, amino and organic acids) has largely contributed to elucidate how and to what extent plant metabolism readjusts to a changing environment [37]. On the other hand, the measurement of specific secondary metabolites such as phytohormones (*e.g.*, auxins, cytokinins, gibberellins, jasmonates, abscisic and salicylic acids), which are key metabolites of signaling and communication between an organism and the abiotic/biotic environment, has contributed to improve our current understanding of the plant growth–defense system [38] (Fig. 3).

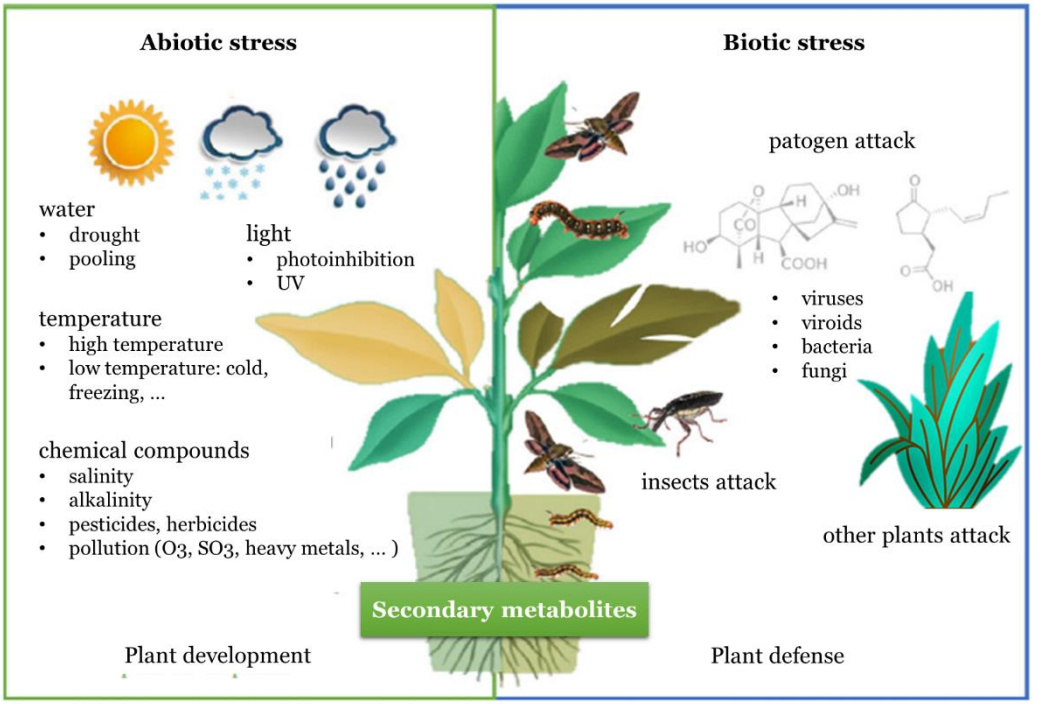

Fig. 3. Abiotic factors (unfavorable environmental factors) or biotic factors (attack of pathogens, parasites, herbivores, etc.) that promote secondary metabolites by plant stress.

Plants, in natural and cultivated ecosystems, are exposed to environmental stresses, biotic and abiotic stresses, along the processes involved in their development. Resistance to the latter can be qualitative or quantitative [39]. Qualitative resistance,

because of monogenic inheritance, has been successfully transferred to elite cultivars to improve resistance; whereas quantitative resistance is generally considered to be durable, no race-specific, and effective against multiple pathogens. Abiotic stresses result from inappropriate levels of environmental factors, such as drought, flood, extreme temperature, severe radiation, metal ion stress, nutrient limitation, and oxidative stress, while biotic stresses come from pathogens and pests.

Advances in plant metabolomics can be applied to improve plant resistance to abiotic and biotic stresses, as once plant receptors are stimulated by stress signals, expression of stress responsive genes is activated and subsequently specialized metabolites (mainly some secondary metabolites) are biosynthesized to adapt to environmental stresses [40].

2.3.2. Metabolites production. A basic difference in the metabolites production in the plant and animal kingdoms is that in the former, metabolites are produced by their cellular biochemical machinery from carbon dioxide and inorganic nutrients, while in animals metabolites are produced from organic and inorganic nutrients in a set of complex processes involving glycolysis, oxidative decarboxylation, citric acid cycle or oxidative phosphorylation.

2.3.3. Number of metabolites. Another big difference is the number of metabolites that exists in each kingdom —higher in plants—, which is a consequence of their very varied functions as a consequence of the defense necessities of the plants. The plant kingdom is routinely stated to contain between 100 000 and 1 million metabolites [41], with any given species thought to contain upwards of 5000 metabolites [42]. Although the core central metabolism of most plant species is largely comparable with that of no plant species, plants and fungi contain a vast wealth of specialized compounds that account for the vast majority of the diversity within their metabolomes. These specialized, secondary metabolites collectively act as an effective arsenal against the myriad of biotic and abiotic stresses to which they, as sessile organisms, may potentially be exposed during their lifespan. Measuring such a vast number of entities is right difficult; however, the problem is exacerbated by the fact that metabolites have highly diverse chemistry [43], a massive dynamic range [42], and are often compartmented at both the cellular and subcellular level [44].

On the contrary, once the first draft of the human metabolome —which can be

considered to be representative of the animal kingdom— has been completed, approximately 2500 metabolites have been cataloged, and 1200 drugs and 3500 food components have also been found in the human body [45]. In fact, it is difficult today to fully estimate the exact number of metabolites in our metabolome considering, for example, the vastly unknown metabolites arising from mammalian gut microflora metabolic cross-talks and so on. Presently, the coverage of the human metabolome is not well known, and similarly happens with plant metabolites, as only a few thousands of them have been measured, whereas between 100 000 and 1 million are estimated to be extant within the plant kingdom (Fig. 4). Even more, of the few thousand metabolites currently assigned either unambiguously or with a high level of confidence, the precise *in vivo* function of many is unknown, and even their spatial abundance is not always clearly understood [23].

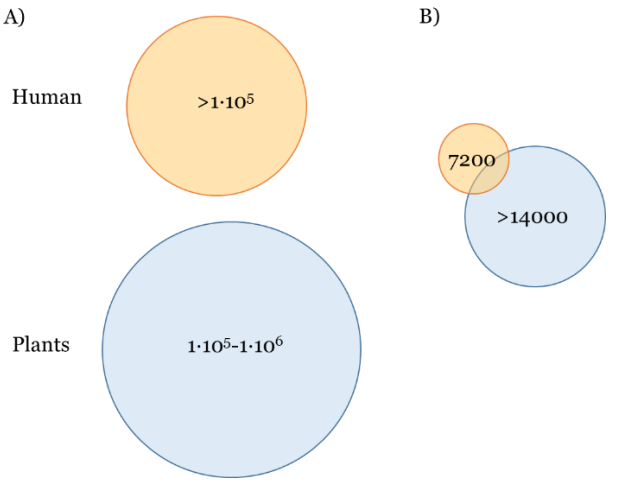

Fig. 4. (A) Estimated number of metabolites in humans and plants. **(B)** Estimated number of metabolites measured so far.

2.4. Other differences between plant and animal metabolomics

Other differences between the two kingdoms that strongly affect the design and development of experiments to obtain information about them are the time, ethical considerations and price for development of the given population to be studied, the sampling and size of the sample, the necessity or no for quenching and for other preliminary steps, and sample preparation. After the analytical sample is obtained, the

other steps involved in the analytical process are common in dealing with the obtainment of analytical information, which, logically, require to be interpreted from the appropriate point of view or final aim of the study. All them are exposed and discussed in the next section.

3. The analytical process as a function of plant or animal metabolomics approaches

Metabolomic analysis consists of four experimental parts (Fig. 5), among which the two first clearly differ and depend on the kingdom to which the system on study belongs. Overally, preliminary operations, which include sampling and sample pretreatment, is the part with the highest differences between kingdoms, whereas sample preparation can be quite similar, most times, but dependent on the metabolomics strategy. Once the analytical sample (a liquid, but less commonly it can be a gas) is obtained, it is ready for being inserted into either the detector or high-resolution equipment for individual separation of the analytes, if required. In the latter case, the analytes are then sent to the on-line connected detector, which provides abundant data to be mined by the chemometric approaches that allow obtainment of the results to give appropriate response to the raised question.

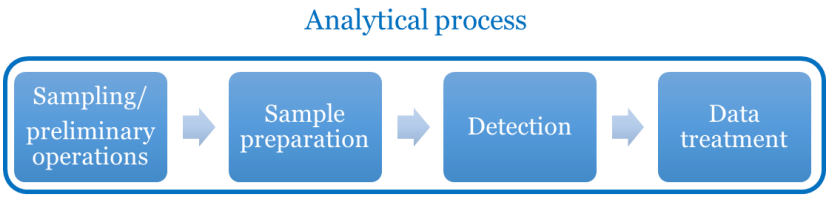

Fig. 5. Scheme of the steps involved in the analytical process.

3.1. Sampling and sample pretreatment as a function of plant or animal metabolomics approaches

The steps that precede sample preparation as such are those that mediate between the system under study and the sample ready for being subjected to sample preparation, which finally will produce the analytical sample. These previous or preliminary steps are

very different depending on the vegetal or animal nature of the system under study; therefore, they are considered separately.

3.1.1. Presampling in plant metabolomic studies. A good experimental design in plant metabolomics heavily depends on the type of system to be studied, which commonly consists of greenhouses, growth chambers, or experimental fields.

(a) In the case of a greenhouse or a growth chamber, the starting point of the experiment, must include, (i) the selection of appropriate growth conditions (*e.g.*, photoperiod, relative humidity, temperature); (ii) a suitable number of biological replicates and controls; and (iii) the use of randomization practices throughout the entire experimental workflow [46,47]. It is also advisable to monitor environmental variations during the progress of the experiment, such as small differences in temperature or light intensity that might occur in the greenhouse or growth chamber; minor variations can greatly affect the biochemical status of the plant [48].

(b) In an experimental field the design involves, (i) selection of the part of the plant to be sampled, which most times is fruit from a given variety or from a number of selected varieties; (ii) the time for sampling (either a fixed time during maturation or growth and maturation evolution [49]); (iii) the sampling design that depends on the characteristics of the experimental field (*e.g.*, a sampling methodology based on random rectangular coordinates [50]); (iv) the number of samples per coordinate point. A study on an experimental field requires repetition for several years to eliminate the characteristics of a given year (anomalous values of pluviometry, drought, cold period, etc.).

3.1.2. Presampling step in animal metabolomic studies. Equivalent to plants presampling, in animal or in human studies this step consists of cohort selection, which can be composed only by people suffering a given health problem or by patients and healthy people (controls). Depending on the level of the planned study (preliminary or validation study) the number of participants can vary from 10–20 to several thousands. Also the number of samples per individual is a function of the longitudinal or transversal nature of the study. Ethical issues must be rigorously completed and the ethical committee of the involved hospital must accept the study.

3.1.3. Sampling, quenching and molturation in plant metabolomic studies. There are several aspects to take into account regarding to the moment in which plants are sampled. The time of the day selected is very important, since the levels of plant metabolites (both

primary and secondary metabolites) vary throughout the day [51]. Strict control of the presampling conditions (both cultivation conditions and sampling regime —time of day, location on the plant, time from sampling to metabolic quenching, etc.) is essential for sample uniformity. Another point to consider is the part of the plant used, since the metabolite content varies throughout the plant. All organs should be separated when possible. Not only does the content vary from organ to organ, but even within the same organ different metabolites might be found. For example, there are large differences between young and old leaves, but even there is a difference between the veins and the rest of a leaf [52]. Sampling of plant materials requires special care because rapid metabolite changes, such as those due to enzymatic degradation or oxidation, could occur during the process and may affect the results drastically. For this reason, sampling (*i.e.*, separation of the required material from the original plant) should be conducted very rapidly.

Quenching consists of fast inactivation of enzymatic reactions in the sampled material, of particular importance to avoid fluctuations in the levels of fast turnover metabolites (*e.g.*, glycolytic intermediates) [53]. Inactivation of the metabolism should be faster than metabolic changes occurring in the sample. The effectiveness of the quenching process is crucial, since the turnover rates of many primary metabolites are usually in the range of 1 mM/s —(taking into account that concentration of metabolites varies from a few molecules per cell, as in the case of certain signaling molecules, to mM concentrations for some primary metabolites (*e.g.*, glucose)— [54].

The common strategies for quenching are based on rapid modification of sample conditions, usually pH or temperature. In modifying pH, quenching is achieved by instantly changing to extreme pH, either to high alkali (*e.g.*, by adding KOH or NaOH) or to high acid pH (*e.g.*, by adding perchloric, hydrochloric or trichloroacetic acid) [55]. In modifying temperature, quenching is mainly carried out by cooling at values usually lower than −20 ºC, assuming that sample integrity is not endangered by the cold shock [56]. The most popular method is cold methanol quenching, which allows a rapid interruption of the metabolism in the sub-second time scale [57] and can be implemented in protocols destined to discriminate between intracellular and extracellular metabolites [57]. Freezing is the most used way of quenching as it helps to stabilize labile metabolites, but tissue excision and freezing need to be accomplished quickly to avoid induction of wounding responses. Rapid freeze methods include immersion into liquid nitrogen, liquid nitrogen–cooled isopentane and freeze clamping. As freezing generally preserves protein structure,

care needs to be taken to avoid warming that may allow some enzyme activity to perturb metabolite pool composition. Freeze-drying (also known as lyophilization) can help to stabilize frozen samples, but may also cause a loss of some metabolites through irreversible binding to cell walls or membranes, and also result in the loss of volatile metabolites; nevertheless, lyophilization positively protects against enzyme activities and microbial decomposition during storage [58]. Care must be taken with lyophilized samples as they can still reabsorb atmospheric water and regain some enzyme activity. The influence of quenching (*viz*., lyophilization or dried under heated air) or the absence of this step (fresh sample) on citrus has been in-depth studied by the group in which the PhD student is integrated [59]. Metabolism has rarely been interrupted by a heating shock with a fast increase of temperature. One example can be the addition of ethanol at 90 ºC [54]. However, this method seems not to be a competitive option owing to potential degradation of thermolabile metabolites and increased cell permeability [60]. Apart from batch protocols, a variety of approaches for automated quenching have been reported [61,62]. These are based on on-line coupling of a fast sampling device to a bioreactor, where the metabolism is stopped.

After quenching, plant tissues should be finely homogenized to obtain a homogeneous powder. This step is critical for plant tissues, in which cells are surrounded by a thick wall that must be breached before metabolite extraction. The most common procedures for plant-cell-wall breakage and homogenization use a mortar and pestle, ball mill, vibration mill, Ultra Turrax, ultrasonic probe or thermomixer. When possible, this step is performed under liquid nitrogen to prevent tissue defrosting. Finally, the powder must be divided into portions (samples) and stored at low temperatures until analysis.

Plant growth metadata documentation and storage in databases is crucial, not only for the ongoing experiment but also for the data reanalysis and the possibility to perform data meta-analyses combining several experiments [63]. Storage conditions have to be controlled, as stability during sample storage is a key factor that is rarely taken into account.

3.1.4. The overall sampled material, pretreatment and sampling in animal metabolomics studies. We restrict here the animal field to the clinical human field as it has received —and is receiving— the highest interest and research resources, and also because this has been the animal field to which part of the research in this PhD-Book has been focused.

3.1.5. Types of biofluids and tissues in clinical metabolomics studies. Among clinical biofluids, blood —plasma or serum depending on allowing or blocking coagulation,

respectively— is the most commonly used in metabolomics studies, fact justified because its minimally invasive sampling, homogeneity as compared to urine, sweat, saliva, etc.; all them strongly influenced by the collected volume. Urine is also a common biofluid in metabolomic studies, while other less common biofluids used in this field are saliva, tears, exhaled breath condensate —see "recent contributions from the group", in section 4 of this introduction—, and sweat, widely investigated by the PhD student (see Chapters VII-to-XI). A key difference among them is the necessity for specialized personnel to obtain blood, and the only necessary consent by the donor in the case of the other biofluids.

Whereas serum or plasma require containers with special characteristics, and to specify the time of the day when it is collected, urine is collected usually in bare polypropylene containers of the required volume, but the time of urine sampling must be planned depending of the target study: random samples, timed samples, 24-h samples, and even longer times for urine collection in dealing with pharmacokinetics studies [64].

Tissues are less common samples because their invasive character; therefore, they are most times restricted to surgical interventions.

Independent of the nature of the target biospecimen, the overall portion removed is subjected to some type of pretreatment: division into the appropriate containers to obtain plasma or serum in the case of blood; buffering, dilution or evaporation, centrifugation and/or deproteination in any biological fluid, before being divided into bare or special containers or vials. This last step could be considered properly as sampling. Then, the samples are, in general, subjected to low temperatures (usually –80 ºC) to quenching enzymatic processes, especially important in saliva, and to store them until use.

3.1.6. Cell-sampling. Special mention deserves cell-sampling approaches that allow metabolites profiling, a promising field still in its infancy. Recent technological advancements —especially those made just in the past four years— have revealed that individual cells within the same population may differ dramatically, and these differences can have important consequences for health and disease. The rapid, dynamic responses a living cell has to its environment are better reflected in the metabolic transformations —and the resulting patterns of small molecules— that keep the cell powered, cycling and communicating with others [65].

3.2. Sample preparation

This step, after which the sample is converted into the analytical sample —that is ready for being introduced into the analytical platform—, strongly depends on two key aspects: the type of sample and its physical state, and the metabolomics strategy that follows sample preparation. The procedure for sample preparation requires to be optimized by one of the chemometric approaches discussed in Section 3.6.

Overall separation of the target metabolites from the matrix consists of an extraction step, the selection of which depends on the aim of the study: extractants and extraction technique must be carefully chosen to obtain the expected results. In dealing with solid samples, the solid–liquid extraction step (properly known as leaching or lixiviation) can consist of maceration, Soxhlet extraction, ultrasound-assisted extraction (USAE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE) or superheated liquid extraction (SHLE), as the most used. Metabolites from liquids are mainly removed from the sample by liquid–liquid extraction (LLE), solid-phase extraction (SPE) or solid-phase microextraction (SPME). Volatile compounds are easily separated from the sample (either solid or liquid) by heating, mainly using a head-space (HS) device.

Ideally, the extraction step aims at: (i) releasing given metabolites from the sample in an efficient manner; (ii) avoiding the presence of interferents that could difficult the analysis; (iii) making the extract compatible with the analytical equipment; and, (iv) concentrating trace metabolites before analysis, if necessary [66]. An example of the key influence of the characteristics of the extractant on the nature of the extracted metabolites is given in Chapter V of this PhD-Book, in dealing with aqueous enzymatic extracts of *Agaricus bisporus*.

3.2.1. Sample preparation in plant metabolomics. Because most of the plant samples are solid, the most common procedure for removal of the metabolites from the sample matrix is solid–liquid extraction in any of its types. In the case of volatile metabolites such as essential oils, separation from the sample matrix is most times carried out by cold press, steam distillation or hydrodistillation, but also by HS or pervaporation. In dealing with juices, the sample is only subjected to centrifugation and/or filtration to give place to the analytical sample.

Maceration or Soxhlet extraction have for many years been used to extract bioactive compounds from plant materials as a model for comparison and validation of

methods based on the use of auxiliary energies. These conventional approaches involve typical shortcomings (*e.g.*, long development times, large amounts of extractants and potential and never checked degradation in the case of Soxhlet). USAE has been applied to obtain metabolites from plants using either an ultrasonic cleaning bath or a probe (each with its advantages and shortcomings [67]), always with drastic shortening of the extraction time from hours to minutes. Wide information on the functioning of ultrasonic devices and their positive and negative aspects can be found in Chapters I-to-III.

Also MAE provides high yields in a short time as compared to traditional methods. Microwaves penetrate the sample and heat instantaneously individual polar components in the matrix, thus achieving extractions much faster than traditional heating methods that depend on the thermal conductivity of the sample [68]. However, microwaves irradiation can accelerate chemical reactions and degradation of thermolabile metabolites and also modify the chemical structures of some compounds like carotenoids [67]. A wider information on this subject can be found in a book chapter by the PhD student, included in the Book as Annex II.

SFE, carried out most times by CO_2 as extractant, was used to remove no polar compounds as essential oils from citrus [69]. In dealing with polar compounds, SFE requires the presence of a polar co-solvent such as ethanol, which complicates the step by increasing both costs and handling. Gases such as ethane have also been used as extractants in SFE of essential oils [70]. The cost of high purity CO_2 makes its use prohibited for extraction of low price compounds, for which cold pressure extraction is preferred at large scale production. Nevertheless, the high selectivity of supercritical CO_2 for extraction of terpenes has led to its use for deterpenation of essential oils [71] previously obtained by cold pressure extraction [72], because of the high commercial value of refined essential oils.

SHLE involves heating of the extractant above its normal boiling point and keeping it in the liquid state by applying the necessary overpressure. Under these working conditions the extractants increase their ability to remove analytes and improve their diffusivity in the sample [73]. The most important factors in dealing with SHLE are temperature, type of extractant and extraction time. SHLE, used to obtain essential oils from different plants [74] and citrus bioactive compounds such as flavonoids [75], offers several advantages over other extraction techniques, namely: possibility of automation, low extractant volumes and decreased extraction time. Implementation of SHLE can be accomplished in the batch mode, in which the sample and extractant are kept into contact

for a preset time at constant temperature and pressure, or in the continuous mode in which the extractant flows through the sample in a continuous manner [76]. The main shortcoming of SHLE in any of its approaches is the working temperature, usually high, that can degrade thermolabile compounds.

In general, degradation of metabolites by auxiliary energies has been understudied, as the research in this aspect has been mainly focused on the effect on a single metabolite or group of metabolites, as is the case with phenols [77]. The scant studies on this aspect have monitored degradation by decrease of the signal provided by the precursors. Based on the decrease the authors concluded that properties such as antioxidant activity and antiradical scavenging, among others, are deteriorated. In-depth studies on degradation products could show that not all degradation reactions are undesirable; in some cases these reactions yield compounds with better properties than their precursors. An example that supports this assertion is the decrease of glycosidic-conjugated flavonoids and increase of the corresponding aglycons in extracts from *Citrus reticulata* pomaces obtained by MAE [68], and extracts from *Citrus unshiu* pomaces treated with electron-beam irradiation, in which the antioxidant power increases from conjugated to free flavonoids [78]. Full understanding of the effect of auxiliary energies on metabolites treatment (either extraction or conservation) requires a simultaneous study of all factors involved in the step and their responses. The results from degradation studies should be based on a global profile of the components resulting from the given step and not only on a specific metabolite or response.

3.2.2. Sample preparation in clinical metabolomics studies. Clinical metabolomics involves mainly liquid samples (serum or plasma, urine, saliva, tears, sweat, exhaled breath condensate, etc.), but there are also protocols for sample preparation from solids such as organ tissues (*e.g.*, brain, liver and tumor tissues), feces or whole organisms (*e.g.*, earthworms). The sample matrix (*viz.,* the whole sample except the target analytes) takes extreme importance in metabolomics analysis because it influences the choice of sample preparation owing to its potential interference in the extraction and/or detection of the target compounds [79]. Regardless of the sample matrix, the different behavior of the target compounds and matrix components ultimately determine the choice of sample preparation.

Depending on the metabolomics analytical strategy, sample preparation can be a very simple step or not. Some examples of sample preparation steps illustrate this assertion: (i) the analytical sample for urine analysis can be obtained by simple dilution, by

centrifugation or by filtration for cell removal. Bacterial growth inhibition or urease treatment (urea removal) can also provide the analytical sample from urine [80]. (ii) Plasma samples can need only anticoagulant selection and protein removal for being an analytical sample; but for targeted analysis, more commonly they require an SPE step that can be developed on-line with the high-resolution separation and detection steps [81], without real handling of the analytical sample. (iii) Serum samples can also need only protein removal, but commonly an SPE is mandatory. Also in this case, the on-line connection of this step (that can involve overall separation and concentration of the target metabolites) avoids sample handling and reduces drastically the sample volume [82]; aspect of great importance in clinical samples, mainly in cases of neonates, elderly people or patients in critical state. (iv) cerebrospinal fluid samples can need only protein removal for being an analytical sample, but frequently they require an SPE step. (v) Tissue samples must usually be subjected to complex sample pretreatment consisting of rinsing to remove blood, quenching, and homogenization, before being subjected to sample preparation, generally an SPE step, which can be followed by evaporation of the eluent and by reconstitution. Evaporation of the extractant or the eluent and reconstitution can also be mandatory for sample preparation in dealing with plasma or serum in the case of very diluted metabolites and/or necessity of change of the medium before entering in the detector or high-resolution separation equipment.

The presence of standards (either conventional or isotopically labelled standards) is highly demanded to ensure reproducible and accurate results. The time for their addition closely depends on the different steps in which the sample is involved and selection of the proper moment is a matter of discussion, most times.

3.3. Detection in the analytical process of metabolomics studies

Once the analytical sample (*e.g.,* an extract, an eluate, a reconstituted solution after evaporation of the solvent, a volatiles mixture) is obtained, the complexity of the next step depends on the required information to be mined from the prepared sample, which in turn establishes the analytical approach to be used. The metabolites to be determined in the analytical sample can be amino acids, lipids, etc., and they can come originally from a plant or animal sample. Therefore, despite the importance of the preliminary and sample preparation steps, detection is —with or without prior high-resolution separation— the step

that provides the analytical information to be then treated as required. The final aim of the study establishes the necessity or not of high-resolution separation and the type of detector, which can provide single or multi-information.

There is a variety of analytical strategies to be used in metabolomics that not all are complex; therefore, the analyst must know what is the most appropriate in each situation and use each as required. From the simplest (unselective analysis) to the most complex (complete identification–quantitation) the necessary analytical platforms (and subsequent chemometric approaches, the subject of the next section) and applications are discussed below.

Unselective analysis of an analytical sample refers to the total content of components capable of being related through a common characteristic. The simplest are total indexes (*e.g.*, measurement of the absorbance of phenols contained in a vegetal extract at 280 nm or that of anthocyanins at 520 nm). A method that is not based on measurement of an intrinsic property of the target compounds is the Folin–Ciocalteu (F–C) method, often used for the determination of total phenols content. The F–C reagent forms colored products with the analytes (usually containing hydroxyl groups) with maximum absorbance at 765 nm. A calibration curve from a single reference standard as gallic acid [83] is used to interpolate in it the absorbance of the extracts. Also well known reagents such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) are used to determine the total scavenging capacity of an extract, while ferric ion reducing antioxidant power (FRAP) allows determining its antioxidant activity [84].

Individual quantitation of a metabolite or a small group of metabolites can provide, sometimes, the necessary information about the state or evolution of a given organism. This information is obtained by a relatively simple analytical platform constituted by a liquid or gas chromatograph coupled to a detector based on molecular absorption [either UV–visible detector or diode array detector (DAD)] or flame ionization detector (FID), respectively. This relatively simple analytical equipment requires standards to identify the target compounds based on the retention time, and also supported on the molecular spectrum in dealing with UV–visible detector or DAD. The absence of commercial standards makes difficult or hinders development of methods and thus the use of this approach [85]. Examples of its use are the individual separation of phenols, carotenoids and vitamin C in citrus carried out by C18 or UP-18 chromatographic columns in an LC coupled to a UV–

visible detector [85]; and that of volatiles from essential oils, quantified in a simple manner by GC–FID with the help of available commercial standards [86].

More complex, common metabolomics studies require detectors with high sensitivity to detect metabolites at low concentrations, high-resolution power to identify them and wide dynamic range to detect them at variable concentrations in complex matrices. The most used types of detectors (MS and NMR) are described in the next subsection.

3.3.1. MS detectors. MS fundamentals is the differential movement of ionized molecules through vacuum by administering an electrical field. A mass spectrometer consists of a sample inlet, an ion source, a mass analyzer, a detector and a system for data collection. The sample inlet let put the sample molecules inside the ion source, where they are ionized. The ion source produces gas-phase ions via an ionization technique, the mass analyzer separates the ions according to their mass-to-charge ratio (*m/z*), and the detector produces an electrical current from the incident ions that is proportional to their abundances, and constitutes the collected signal.

Fig. 6 shows the main ionization sources and types of mass spectrometers, as well as the types of high-resolution separation approaches to which these detectors can be coupled. All them are discussed below.

In dealing with ionization sources in MS, the most used, as summarizes Fig. 6, are electrospray ionization (ESI) for coupling to liquid chromatographs (LC–MS) and electron impact (EI) ionization when the coupled chromatograph is a GC (GC–MS). ESI is the most widely used for untargeted metabolomics studies, because it produces soft ionization (*viz.*, ions are generated with little or without fragmentation, which can support the identification of unknown metabolites) and can ionize compounds within a wide range of polarity and mass. The produced ions are adducts with proton or other species present in the mobile phase. It is a very useful property to determine compounds such as acids, amines, proteins and peptides [87]; and, in general, this ionization source is useful to study polar volatile and no volatile compounds, thermally stable and no stable compounds, and polar compounds. An example of parameters optimization of this source for quantitation of amino acids can be found in Chapter VII of this Thesis-Book.

In addition to the wide use of ESI, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) have been used to receive the

eluate from LC, CE and supercritical fluid chromatography (SFC). APCI is of interest for ionization of carbohydrates, triglycerides, amines, esters, and phenols, and as complementary source of ESI, because it allows determining no polar compounds. APPI is a useful alternative to study compounds that are poorly ionized by ESI or APCI sources. EI, which consists of interaction of the gas molecules with kinetically activated electrons at an energy of 70 eV, and chemical ionization (CI) sources are frequently used to couple the mass detector to GC equipment. Both sources are complementary because EI allows obtaining structural information and CI molecular mass information, thus enhancing characterization.

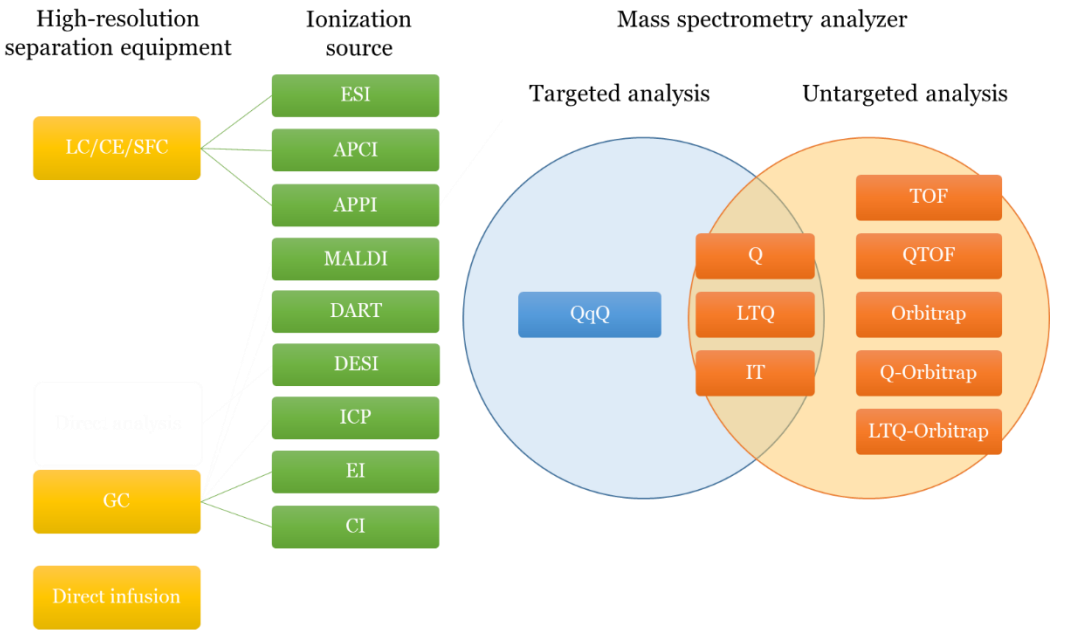

Fig. 6. High-resolution separation approaches, ionization sources and MS analyzers more used in metabolomics.

Other interesting source is matrix-assisted laser desorption/ionization (MALDI), normally coupled to time-of-flight (TOF) equipment for protein analysis, and for lipidomics studies. Surface-enhanced laser desorption/ionization (SELDI) is a variation of MALDI widely used to identify new protein molecular biomarkers. MALDI can be used

independently or coupled to LC. Other ionization sources of MS used in metabolomics studies are direct analysis in real time (DART), inductively coupled plasma (ICP) and desorption electrospray ionization (DESI). Mass analyzers can be used either alone or combined with the same or different type of mass analyzers [hybrid instruments or tandem MS (MS/MS)]. Tandem MS and multiple-stage MS favor identification of a given molecule because the molecular ions and fragments from precursor ions can be detected.

Concerning the characteristics of mass analyzers, the most remarkable are as follows: (a) mass accuracy of the measured *m/z*, related to the mass resolving power and stability of the instrument. (b) Mass resolving power or ability of an MS to provide a specified value of mass resolution (*i.e.*, the instrument generates distinct signals for two ions with a small *m/z* difference). (c) Mass range or limits of *m/z* within which an MS can detect ions or operate to record a mass spectrum. (d) Transmission efficiency or ratio of the number of ions reaching the detector and the number of ions leaving the mass analyzer, related to the sensitivity of the mass spectrometer (*i.e.*, the minimal concentration of a compound leading to a peak intensity greater than a specified signal-to-noise ratio). (e) Scan speed or rate at which the analyzer measures over a certain mass range. (f) Scan cycle time or the time required to obtain a mass spectrum, also known as duty cycle.

The selection of a given MS detector depends on the type of metabolomics analysis to be performed, targeted or untargeted, as shows Fig. 6. Mass analyzers for untargeted metabolomics must provide the appropriate mass resolution power, mass range and mass accuracy. TOF, quadrupole time-of-flight (QTOF), Orbitrap, Q-Orbitrap and linear ion trap (LTQ)-Orbitrap are used in this context, and, among them, TOF and QTOF instruments are preferred because they provide data acquisition over a wide mass range with high mass accuracy and resolving power, whereas mass analyzers have to provide transmission efficiency, sensitivity, scan cycle time and scan speed to be used for targeted metabolomics —QqQ is the most useful instrument in this case. Other instruments such as single quadrupole (Q), ion trap (IT) and LTQ are used in both targeted and untargeted studies.

MS has been used both to implement plant metabolomics strategies [88,89], and to develop and enhance clinical metabolomics assays in toxicology, endocrinology and biochemical genetics areas.

The use of MS alone is called direct infusion mass spectrometry (DIMS). Its main disadvantages are the high-resolution mass analyzer requirement (more expensive

instrument) and ion suppression effects (more difficult identification and quantitation of low concentrated or difficult of ionizing metabolites).

3.3.2. NMR detectors. NMR spectrometry is another prevailing technique in metabolomics thanks mainly to its resolution capabilities. Briefly, in NMR detectors, liquid or solid samples are placed inside a detection coil, and exposed to an external, static and homogeneous magnetic field referred to as Bo (*z*-axis). The magnetic moments in the sample align along B_o according to a Boltzmann distribution. According to quantum mechanics, magnetic moments can only align parallel or anti-parallel with respect to this external field, leading to a macroscopic magnetic moment M. After a short application of a high frequency B1 field orthogonal to B_o (B), the magnetization is aligned with the rotating B1 field. Detection of NMR signals can be only from atomic nucleuses possessing unpaired number of electrons (responsible for the resultant spin of these atoms, which align parallel or anti-parallel to the applied magnetic field), property termed as paramagnetism. In the absence of magnetic field, nucleuses are rotating with randomized trajectories, but if a suited magnetic field is applied these nucleuses are aligned with it.

Most elements have at least one isotope with a magnetic spin number greater than zero, necessary for the NMR effect. In the case of biological samples, the two more common nucleuses, ^{1}H and ^{13}C, give place to the two most frequent NMR techniques, ^{1}H-NMR and ^{13}C-NMR. The former is the most sensitive of them because of the natural presence of this nucleus. Thus, magnetization of hydrogen atoms experiencing a magnetic field of 14.1 T will rotate at 600 MHz aligned with the magnetic field. Such a spectrometer is called a 600 MHz detector. This is the reason why NMR spectrometers are usually classified in frequencies. With the same magnet, a ^{13}C spin will process at about 150 MHz due to the natural presence of this nucleus (1.1%). Nowadays, there are available NMR magnets up to 1 GHz that operate at magnetic field strengths of 23.5 T and proton NMR frequency of 1000 MHz with tremendously high field stability.

An NMR spectrum is composed by spectral amplitude plotted *versus* chemical shift. This method, called 1D-NMR, allows determining the composition of a sample only if there is no severe overlap of signals. A method called 2D-NMR is used to overcome this limitation. It consists of a sequence of B1 pulses under systematic variation of an inter-pulse delay, leading to a 2Dmap where the amplitude is plotted as a function of two frequency dimensions. A cross-signal in this map indicates a pair of spins with a transfer of magnetization during the pulse sequence. This allows determining related signals of the

spectrum (such as signal corresponding to the same molecule). 2D-NMR spectrometry can encompass homonuclear and heteronuclear methods if the couplings are between the same or different nucleuses such ^{1}H-^{1}H and ^{1}H-^{13}C, as the most frequent couplings.

The great advantages of NMR are the potential for high throughput fingerprinting, minimal requirements for sample preparation, and the no discriminating and no destructive nature of the technique. In fact, ^{1}H-NMR spectra can be acquired rapidly in 3-15 min with minimum sample preparation that usually just entails buffering and internal standard addition [90]. Furthermore, ^{1}H-NMR has been largely used to unequivocally determine metabolite structures and, perhaps due to its no invasive nature, is more commonly used in mammalian systems than MS technologies. However, only medium-to-high abundance metabolites will be detected with this approach and the identification of individual metabolites based on multivariate analysis of signals that cause sample clustering is difficult in complex mixtures. Indeed, the ^{1}H-NMR spectrum of a complex sample is composed of several hundred resonances (with chemical shifts between 0-10 ppm), which are estimated to arise from a hundred of more metabolites, resulting in a highly congested spectrum, given that each compound usually provide more than one signal each with a certain multiplicity. On the other hand, the reduced sensitivity of ^{13}C-NMR has practically limited its application to the study of metabolic fluxes in cells and tissues by suited fed with isotopically labeled metabolites, thus given place to fluxomics.

Limitations of 1D-NMR have been partly overcome by the use of 2D-NMR and by ultrahigh-field NMR spectrometers, which allow increasing spectral dispersion and reduce strong coupling associated distortions, but highly increase the analysis time. They are used for global profiling analysis.

It is generally accepted that sensitivity in ^{1}H-NMR is lower than in MS. This depends on the magnetic field strength, with higher field magnets providing greater signal-to-noise ratio. This ratio can be increased by increasing acquisition time, which means increased number of scans. On the other hand, integration can be directly carried out by comparison with an internal standard by simple integration. Although NMR can be coupled to LC and SPE columns to improve separation and sensitivity [91,92], it is unlikely that NMR will attain the sensitivity of MS.

Despite the advantageous aspect of 2D-NMR spectra to spread overlapping resonances into a second dimension by the various alternatives to carry out 2D-

measurements, the main limitation of these experiments is the high acquisition time required, typically within 16-20 h to achieve a comparable sensitivity to 1D spectra. Therefore, identification in complex mixtures is still more difficult than in MS and requires large user interpretation. This is the reason why the use of 2D-NMR for metabolomics is usually restricted to the characterization of compounds unidentified from the 1D spectra.

Among the remarkable applications of ^{1}H-NMR fingerprinting with multivariate analysis in plant metabolomics is the identification and classification of maize seeds obtained from transgenic plants into different classes according to changes in metabolites [93], a genetic study of strawberry fruit quality, a functional study of tomato transformants and a study of *Arabidopsis thaliana* phosphoenolpyruvate transformant metabolites [94].

NMR fingerprinting has been used for many years to authenticate foodstuffs, especially in the beverage industry and grape [95]. Using standards methods of ^{1}H-NMR data collection, followed by principal components analysis (PCA) and partial least squares (PLS) methods, predictive modelling pinpointed the spectral areas responsible for a separation according to vintage.

Even with the improved resolution of 2D-NMR approaches, complete characterization of complex mixtures such as plant extracts by NMR is often impossible. Hyphenating NMR to LC alleviates some of the problems by allowing NMR data to be collected on individual comments of a mixture, as in the screening LC–NMR profiles of crude lipophilic extracts of aquatic plants for potential algacides [96]. Nevertheless, the compounds of interest could not be completely identified from the LC–NMR analysis. The lack of enough sensitivity of on-flow LC–NMR for phytochemical analysis is overcoming by previous automated SPE peak trapping, which has been used to investigate the composition of an African medicinal plant, *Kanahia ianiflora* [97].

In the clinical field, and despite its relatively low sensitivity, targeting NMR analysis has been widely applied. In fact, quantitation of biomarkers related to diseases or human metabolism [98,99], including schizophrenia, multiple sclerosis, diabetes, organ rejection, and rheumatoid arthritis have already been published and exploited. Interestingly, NMR-based serum-lipoprotein quantitation is a mainstream clinical diagnostic tool, as also does *in-vivo* NMR analysis, probably the most outstanding application of NMR in metabolomics due to its use in diagnosis of diseases such as cancer. The *in-vivo* NMR “metabolite mapping” of tissues after biopsy or before local ablative

procedures provides precise differentiation of cancer and benign tissue based on detection of given metabolites such as choline [100].

Clinical metabolomics fingerprinting by NMR has proved to be an excellent approach to discover metabolic changes associated with disease in humans [101].

Currently, clinical NMR-based metabolomics allows semi-automated identification and quantitation of metabolites with concentrations as low as 1 μM. This means that up to 20 different metabolites can be detected (*in-vivo*) in certain tissues and up to 100 metabolites (*in-vitro*) in certain biofluids. Given that the metabolome of many biofluids and tissues is of the order of thousands of metabolites [45], a major future challenge will be to find ways of increasing that number. In addition, provided that quantitative NMR metabolomics is carried out almost exclusively in aqueous conditions, it is not surprising that most of the quantifiable metabolites are water soluble, thus limiting considerably its applicability. In addition, no internal standard addition, apart from D_2O (to serve as a frequency lock signal) and a chemical-shift reference standard [sodium trimethylsilylpropanesulfonate (DSS) or trimethylsilyl propionate (TPS)] are required.

It is worth remarking the use of quantitative NMR for *in-vivo* applications. This approach was used for the first time to quantify brain metabolites [102] (glutamine, glutamate and gamma butyric acid) in patients suffering from epilepsy, which were proved to increase as compared to controls [103]. Also, precise quantitation of the major brain antioxidants (vitamin C and glutathione) at μM levels has been described, taking into accoung that altered antioxidant profiles in the brain have been linked to schizophrenia [104]. Analysis of N-acetyl aspartate, creatine, choline and myo-inositol concentrations in the spinal cord have shown significant differences from those found in the brain stem. This may have implications in the diagnosis of multiple sclerosis and the monitoring of spinal cord injuries, among other diseases [105].

3.3.3. Analytical platforms based on high-resolution separation approaches and MS detectors. Mass detectors have traditionally been connected to chromatographs and, in a lesser extent, to CE devices. Coupling of liquid chromatographs to NMR detectors is more recent and scanter. GC–MS allows profiling a wide range of metabolites with different physico-chemical properties such as amino acids, carbohydrates and organic acids, with a characteristic high reproducibility thanks to the ionization source normally used: EI [106]. The main disadvantage of this approach is its limitation to thermally stable metabolites

that are volatile and/or easy to convert into volatile products. Therefore, the study of thermolabile and/or no volatile metabolites such as sugar phosphates or large oligosaccharides is mainly afforded by LC–MS approaches [106].

Regarding the type of mass detector coupled to GC, a Q/MS (GC–Q/MS platform) provides sensitive, cheap and reliable analysis, but operates at slow scan rates and provides low mass accuracy. When the study deals with compounds identification or comparison among samples obtained in —or subjected to— different conditions, the most appropriate detector coupled to the separation equipment is TOF. GC–TOF/MS provides high mass accuracy, high duty cycles and fast acquisition times. In addition, it permits accurate deconvolution of overlapping peaks, which are common in plant extracts [107]. A complementary approach is GC–QTOF that provides MS/MS information on the structure of the target metabolites and their fragmentation pattern (precursor ion, product ions, loss of neutral mass, adducts formation), which permit identification supported on the appropriate databases. The evaluation of the metabolite profile by the mandatory chemometric approaches makes possible to evaluate the variances among samples, usually as compared with control samples (no treated samples). The necessity for mass standards, which depend on the given instrument and the ionization mode, together with the special training required for data interpretation, and the acquisition cost of these instruments make them prohibitive for low-medium budget laboratories. GC-QqQ has been recently applied in metabolomics, providing accurate quantitative metabolite data [108]. It can monitor simultaneously a large amount of MS/MS transitions, improving the sensitivity and selectivity of the analysis, thus enhancing the quantitative profiling of metabolites [108].

Recent applications of GC–MS platforms (particularly GC–TOF/MS [107], and GC–QqQ MS/MS [108]) prove their importance in plant metabolomics studies, despite the need for derivatization required most times for plant primary metabolites [85,86].

In dealing with LC–MS platforms (more recent than GC–MS platforms because of the shortcomings derived from the insertion of the eluate from the LC column and the necessity for evaporation of the eluant before ionization) their use has complemented (and in countless cases surpassed) that of GC–MS platforms. The demonstrated exceptional performance of LC–MS interfaces avoids derivatization (as compared with GC–MS), and offers a wide range of possibilities concerning columns and mobile phases both for polar and no polar compounds, in addition to the high sensitivity and selectivity of the detector,

mainly in the case of triple quadrupole instruments. This platform is very appropriate to profile a set of known metabolites or compound classes; that is, for target metabolomics analysis. As commented before, the most used ionization source in LC–MS is ESI.

Concerning the different types of mass detectors coupled to LC, QTOF has been used for LC–MS analysis of plant and animal metabolomics, but also QqQ, IT, Q-Orbitrap have been coupled to LC for development of these studies. LC-QqQ is required for quantitative targeted analysis because it provides high sensitivity using the multiple reaction monitoring (MRM) mode, allowing quantitation of very low abundant metabolites [109]. The analysis of metabolites in human body fluids constitutes a challenge because of their chemical diversity and dynamic concentration range, to which LC–MS/MS faces by methods for quantitative analysis and profiling of complete metabolites classes in a biofluid over a broad dynamic concentration range. In fact, LC–MS/MS-based metabolomics approaches in clinical applications aims at both, the improvement of diagnostic sensitivity and specificity by profiling a metabolite class instead of determination of a single metabolite, and identification of new disease specific biomarkers [110].

Especial mention deserves the coupling of supercritical fluid chromatographs to MS detectors. Despite SFC is not a novel technique as it appeared at the beginning of the 90's in the last decade of the XX century together with SFE, the improvement in its different parts (particularly pumps) has propitiated its relatively recent use, and its coupling to MS detectors.

Despite in SFC the mobile phase is a fluid, typically carbon dioxide that is maintained beyond its critical point, the addition of an organic solvent (usually 2–30% of methanol or ethanol) to increase its polarity leads to specific conditions referred to as subcritical. Significant improvements in both instrumentation and column technology have shaped SFC as very promising and competitive in high-throughput analysis due to the specific features of the supercritical/subcritical fluid allowing for better solubility and faster analyte transport without excessive backpressure generation. Higher linear velocities of the mobile phase can be used in SFC due to a less mass transfer resistance explained by the relatively lower mobile phase viscosity and higher analyte diffusivity [111,112].

Similarly to high-performance liquid chromatography (HPLC), SFC made a considerable step forward in bioanalysis upon the introduction of state-of-the-art dedicated or hybrid systems with adapted pumps and backpressure regulators [largely

inspired on developments achieved for ultra-high-performance liquid chromatography (UHPLC)], which are also compatible with modern LC stationary phases [111,112]. Ultra-high performance supercritical fluid chromatography (UHPSFC) exhibits a high kinetic performance with a shifted optimum linear velocity towards higher values (3–5 fold) compared to UHPLC, allowing for ultra-fast analysis. This is an essential advantage in metabolomics studies, particularly considering the low solvents consumption [111,112].

In a lesser proportion than to LC, MS has also been coupled to SFC, most times for lipidomics studies as this type of chromatography is very efficient for lipid class separation [113]. One of the most promising fields of SFC in the context of metabolomics is probably the analysis of isomers such as stereoisomers, diastereomers, and enantiomers. Also UHPSFC–MS platforms have shown their suitability for analysis of medium- and high-polar metabolites with high-throughput metabolomics purposes [114].

CE–MS is still less used in metabolomics owing to poor reproducibility of migration times, absence of interfaces for CE–MS coupling that provide sensitive, robust and stable CE–MS setup and the scant versatility of these interfaces [115]. Neutral compounds can only be separated by specific methods based on addition of SDS, cyclodextrins or micelles formation; reagents incompatible with MS. Nevertheless, CE–MS allows enhancing metabolite coverage, specially of polar compounds. In fact, the metabolome fraction mapped by CE–MS is not usually covered by other platforms [115]. CE provides complementary information to hydrophilic interaction liquid chromatography (HILIC) and also better peak shape. In fact, CE is one of the most suited techniques for separation of phosphorylated metabolites, amino acids or metabolites from the Krebs cycle. Also, isomeric compounds such as citrate/isocitrate or leucine/isoleucine can be easily baseline separated. So, CE has been used for metabolomics studies of diseases such as migraine, schizophrenia, Huntington disease, cancer and chronic alcohol abuse [116,117] .

The combination of some of these approaches, such as NMR, GC–MS and LC–MS, allows identification and quantitation of a big quantity of metabolites. A key example is the research from Wishart *et al.*, who identified 3564 metabolites in human serum [118], by five metabolic profiling methods [NMR, GC–MS, lipid mediators by LC–ESI-MS/MS, lipidomics profiling via thin layer chromatography (TLC)/GC–FID-MS and direct fluid injection (DFI)-MS/MS] to experimentally characterize as much of the known serum metabolome as possible. NMR spectroscopy was able to identify and quantify 49 compounds, GC–MS was able to identify 90 and quantify 33 compounds, lipid mediator

profiling (targeted ESI-MS/MS) identified and quantified 96 compounds, TLC/GC–FID-MS identified and quantified 3381 compounds, while DFI MS/MS identified and quantified 139 compounds. Some of these compounds were identified by several platforms.

3.3.4. Chemical derivatization as an essential or additional sample preparation step to obtain the analytical sample for GC–MS or LC–MS. Derivatization of the target metabolites can be a mandatory step (as is the case of no volatile compounds to be inserted into a GC) or an additional step desirable to improve sensitivity and/or selectivity that can be used either in GC and LC.

In dealing with GC separation, while some plant metabolites such as essential oils are highly suited to be injected into the chromatograph without derivatization, chemical derivatization is usually required to reduce the polarities of the functional groups, facilitate their separation and influence mass spectral properties. To reduce polarity, organic acids are esterified by reaction with diazomethane, but mainly by silylation [119] — predominantly trimethylsilylation (TMS) or *tert*-butyldimethylsilylation (TBDMS). In addition, keto-(oxo-) groups are usually oximated to improve their GC properties and prevent enolization reactions that can introduce multiple products, thereby complicating the chromatograms. In high resolution chromatography, the *syn*- and *anti*- isomers of the oximes can sometimes partially separate giving rise to recognizable shoulders on the GC peaks. As the number of derivatized groups increases, there is the danger in MS detection that the molecular mass of the derivative is outside the mass range of the detector, typically *m/z* 650–1000, or is too high that the derivative will not pass through the GC column. In addition, the likelihood of sterically hindered groups can lead to the formation of multiple products, thereby complicating the chromatogram. TBDMS has been evaluated as the most comprehensive for metabolite profiling applications [120] and it has as further advantage the lower sensitivity of its derivatives to the hydrolytic effects of moisture than the corresponding TMS derivatives during the TMS process, which, in addition, are susceptible to artifact formation. The former give often mass spectra with abundant fragment ions (*m/z* M-57, where M is the molecular mass of the metabolite) from easy loss of the *tert*-butyl moiety, convenient for structural assignments of plant metabolites [121]. A disadvantage of TBDMS derivatives lies in the significant increase in molecular mass, particularly where multiple derivatizable groups are present in the molecule. In addition, problems of steric hindrance might lead to mixtures of fully and partially derivatized analytes. In the study of detailed metabolic processes, particularly where the intermediate compounds may be

reactive or unstable, the analyst should always be aware of such possibilities when interpreting the results. In case of any doubt, alternative derivatization procedures are generally available for specific functional groups [122].

Compared with GC–MS, a major advantage of LC–MS with atmospheric pressure ionization (API) has been the avoidance of a requirement to derivatize the target metabolites in the samples; therefore, the combined approach is, and has been for years, being applied in metabolomics profiling [123] with appropriate choices of column polarity and mobile phase compositions. As derivatization by definition induces chemical changes and is sometimes performed under harsh conditions with a consequent danger of artifact formation, to avoid this step when possible is advisable.

Nevertheless, the literature contains many examples where derivatization can be advantageously employed to enhance the signals obtained from soft ionization MS approaches such as fast atom bombardment (FAB), thermospray, ESI, and MALDI. Analytes in the ESI region containing different functional groups can have diverse ionization properties, giving preferentially positive or negative ions depending on the mobile phase composition. For example, the ESI method in positive mode would work particularly well with positively charged (or easily chargeable) species (highly polar) so that a derivatization step that introduces such species to a wide variety of compounds would facilitate simultaneous measurement (profiling) of an increased number of compounds in one analysis. The same applies to negatively charged (or easily chargeable) species.

Although atmospheric pressure LC–MS/MS is eminently suited to the profiling of polar compounds without such derivatization, applications of derivatization are increasing, usually for specific applications. For example, the clinically important groups of acylcarnitines and amino acids are commonly *n*-butylated prior to direct ESI-MS/MS analysis [124]. The IT MS/MS spectra of the butyl esters of acylcarnitines yield more fragment ions than the underivatized compounds giving more detailed mass spectral 'fingerprints' and thereby increasing their utility for identification [125]. Interesting derivatives based on electrochemically-generated ions can enhance detection characteristics. Ferrocene-based electrochemically ionizable derivatives facilitate analysis of simple alcohols, sterols and phenols [126] and provide further structural information. Alcohols could be selectively detected at low levels in a complex fruit extract after ESI [127]. Fatty acids have been analyzed after ESI using alkyldimethylaminoethyl derivatives [128]. Polar derivatives have been applied to steroids, fatty alcohols and alcohol ethoxylate

surfactants in wastewater, carbonyl compounds, and drug impurities with improved detection [129,130]. In MALDI-MS, charged tris(2,4,6-trimethoxyphenyl)phosphonium (TMPP) derivatives were used for derivatization of peptide amino groups, yielding improved sequence information. Such derivatives have since been applied to amines and carboxylic acids, alcohols, aldehydes, and ketones, oligosaccharides, and carboxylic acids [131,132]. The chromatographic properties of the TMPP derivatives of amines have also been investigated, and a preliminary study has been carried out on the utility of TMPP derivatives for metabolic profiling [133]. Also, electron-capturing derivatives have shown to facilitate greatly the analysis of small molecules at attomole levels [134] with application to lipidomics [135].

The application of stable-isotope-labelled derivatives is having an enormous impact on protein analysis (isotope coded affinity tags, ICAT), a clear indication of the potential utility of chemical derivatization (tagging) in API MS.

Derivatization has also been applied in the research that conform the major part of this PhD-Book for increasing the identification of *Cannabis* compounds by reducing the polarities of their functional groups before *Cannabis* extract analysis by GC–TOF/MS (Chapter VI) and also for increasing the identification of sweat compounds by GC–TOF/MS (Chapter VIII-to-X).

3.4. Data treatment in metabolomics studies

Chemometrics, a chemical discipline that uses mathematical, statistical, and other tools to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analyzing chemical data [136], is essential for metabolomics development. This discipline covers from design of experiments, through data preprocessing and processing, to data analysis. It has been used as tool to design, process, visualize, explore and analyze metabolomics data.

Data analysis strategies used in metabolomics are designed as a function of the approach selected, being different for targeted and untargeted analysis (Fig. 7). The huge amount of data generated in untargeted metabolomics analysis is evaluated by biostatistics and bioinformatics tools.

The main difference between plant and clinical metabolomics analysis is that in the latter the chemometrics treatment of the data can be much more complex, tedious and slow

because of the number of samples that can involve. Although clinical metabolomics studies are frequently based on small sample sizes, representative, well-supported clinical epidemiological studies involve more than 1000 samples. As a way of example, a recent study on the association between serum 25-hydroxyvitamin D and mammographic density, and to assess whether this association differed according to women characteristics, involved more than 1400 premenopausal Spanish women [137].

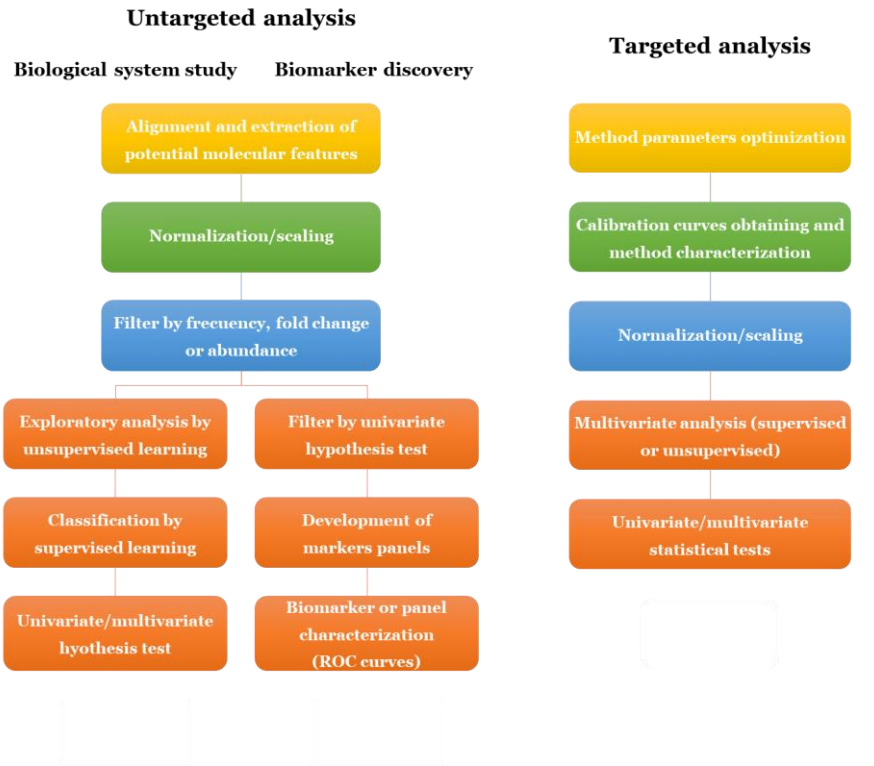

Fig. 7. Workflow of data analysis strategies for untargeted and targeted analysis in metabolomics.

3.4.1. Data treatment for untargeted metabolomics analysis. Untargeted metabolomics analysis consists of covering the maximum number of metabolites using analytical platforms. In this case, the optimization of experimental parameters to obtain the best working conditions for the determination of given metabolites is not required. So, general working conditions are used for untargeted metabolomics analysis. This step covers from data acquisition, through data pre-processing, data pre-treatment to statistical analysis.

Data pre-processing and data-pre-treatment are crucial quality results [138]. The former allows transformation of the raw data into clean data to process them by a data processing software. Some examples for MS data are: extraction of potential molecular features (potential metabolites), finding compounds by chromatogram deconvolution (for GC–MS data), alignment of the signals corresponding to the same metabolites, removal of redundant information and management of missing values. In this step, many factors such as adducts, peak shape, noise removal, etc. need to be considered. Once data pre-processing has finished, the data pre-treatment step (which consists of applying mathematical operations to facilitate comparison among samples) starts. To favor obtainment of information on the relevant biological variations, the experimental and instrumental variables effects should be reduced. For that, the dataset is pretreated to properly transform the data for analysis by using the following tools:

- Normalization, which removes unwanted variation among samples allowing their quantitative comparison. Different normalization approaches as single or multiple internal/external addition, total area normalization or standard to logarithmic transformation can be applied to remove variations among samples.

- Scaling, which consists of dividing each variable by a different factor for an equal treatment of all variables, regardless of their intensity. For example, the mean of each variable is one of the most used factors; while Z-transform can be used in case of combining data obtained from different platforms or methodologies [139].

The use of filters is required in untargeted analysis to reduce the number of potential molecular features and facilitate further statistical analysis. Only potential entities with high intensity, high significance with respect to a factor of interest, or entities that appear with high frequency in the samples batch would pass the filter. The types of filters to be used as required are as follows:

- Filter by abundance, which consists of removing all potential molecular features below a certain abundance.

- Filter by frequency, which consists of eliminating entities present below a certain percentage of samples from each group.

- Fold change analysis, which consists of comparing the level of each potential molecular feature among the groups under study and retaining only the entities that show a preset change.

- Filter based on univariate hypothesis test, which consists of eliminating entities with a *p*-value above 0.05 or 0.01.

- Filter by Volcano plot, which is a combination of analysis of variance and fold change analysis.

The large multivariate data sets generated by this type of metabolomics experiments constitute a great challenge from the point of view of statistical analysis. The outputs of a metabolomics data analysis may differ greatly depending on the purpose of the research. Untargeted metabolomics studies can be divided into two categories —those that aim at understanding biological processes and those that aim at discovering biomarkers [140]. In the first case, an extended set of compounds may be desirable when a biochemical network is under examination, while in the second case, very few highly dependable metabolites can suffice for diagnostic purposes. The second case has not been as widely applied in plant metabolomics studies as in the case of clinical metabolomics studies.

i) Untargeted metabolomics analysis to understand biological processes. The study of the influence of a target factor in a batch of samples and the identification of the most affected molecular features can be performed by different strategies. Usual chemometric tools [141] such as PCA [142] are generally used for display and exploratory analysis purposes, whereas univariate statistical tests such as the Student's *t*-test are used for identification of the relevant variables after exploratory analysis.

- Exploratory analysis by unsupervised learning: unsupervised methods try to analyze a set of observations without measuring or observing any related result.

No specified class label or response exists, so the data set is considered as a collection of analogous objects. Unsupervised learning employs procedures that try to discover the natural partitions of patterns to simplify the understanding of the relationship among the samples and to highlight the variables that are responsible for these relationships. By providing means for visualization, unsupervised learning aids in the discovery of unknown but meaningful categories of samples or variables that naturally fall together. The achievement of such approaches is, in general, evaluated subjectively by the interpretability and usefulness of the results with respect to a certain problem.

PCA is a general no supervised approach, an orthogonal transformation of multivariate data first formulated by Pearson [143] generally employed as an exploratory analysis by extracting and displaying systematic variations and visualization tools to detect

trends and/or groups and outliers. PCA attempts to uncover hidden internal structures by constructing principal components describing the maximal variance of the data [144]. It is a very suitable tool for displaying purposes as it provides a low-dimension projection of the data by transformation into a new different coordinate system preserving as much of the relevant information as possible while discarding the noise. The basic concept relies on areas of signal variance in the data where underlying phenomena can be observed. This principle leads to a focus on a minor number of uncorrelated independent signals that explicate a great part of the total variance in a compact and insightful mode. Practically, PCA creates hyperplanes in the original space that are linear combinations of the original variables and delineates the data in a least squares view. The PCA scores and loadings plots inspections highlight the associations among the distribution of samples that may reveal grouping, trends or outliers and the corresponding variables. More successful data analyses can be achieved on the reduced dimensional space, such as clustering, pattern recognition or classification. Most of metabolomics studies comprises PCA as a first exploratory step [145,146].

- Classification by supervised multivariate analysis: supervised learning contemplates each object with respect to an observed response and includes regression and classification problems depending on the output type under consideration. The objective is to study the mapping from the first to the last. For that, this classification aims at generating general hypotheses based on a training set of examples that are defined by several variables and identified by known labels corresponding to the existing classes.

Several approaches have been developed for that purpose, based either on statistics or on artificial intelligence. Within the diversity of supervised statistical approaches, the most employed is PLS, but they include also decision trees, artificial neural networks and support vector machines. This approach is useful where less observations (N) than measured variables (*e.g.*, detected features, K) are available. PLS use has becomes popular because its ability to deal with many correlated and noisy variables creating megavariate data structures (K>>N) [147].

PLS forms a low dimensional sub-space based on linear combinations of the original X-variables and facilitates the use of the additional Y information by adjusting the model to capture the Y-related variation in X. A PLS-based classification thus has the property to construct data structures with an intrinsic prediction power, by maximizing the covariance between the data and the class assignment. The decomposition relies on latent

variables that are computed sequentially to offer a good correlation with the remaining unexplained fraction of Y. In a classification context, PLS discriminant analysis (PLS-DA) is done to sharpen the partition between groups of observations, in such as way that a maximum separation among classes is achieved. Then, the model can be analyzed to understand the variables that carry the class-separating information. PLS-DA was confirmed to be a powerful tool for classification of metabolomics data [148].

- Univariate hypothesis testing: the Student's *t*-test, the one-way analysis of variance or the no parametric equivalents can be employed for the identification of statistical differences among samples of distinct classes. The predictive power of each variable is evaluated by finding statistically significant differences between the mean intensity values of a given signal, of which the calculated *p*-value is a straightforward indicator. Such procedures are comprehensible but their use is rather restricted in dealing with thousands of highly correlated variables. False positives (type I error) are possible to happen when performing multiple comparisons. Procedures such as the Bonferroni of the Benjamini correction have been implemented to address this issue [149]. Most of metabolomics dedicated software provides statistical hypothesis testing [150,151].

ii) Untargeted metabolomics analysis to discover biomarkers. Biomarker discovering must be made before deriving a definitive multivariate predictive model. Between 1 and 10 biomarkers yield mathematically robust and useful models for clinical testing purposes. Normally, supervised machine learning algorithms (PLS), or multivariate regression models are used for modelling the discriminatory relationship between a binary dependent variable and one or more explanatory variables.

Regarding the selection of potential biomarkers, iterative methodologies for biomarker panel development, such as the used in the PanelomiX bioinformatics tool or in the ROCCET on-line tool, are recommended.

Biomarker panels are designed to discriminate with an optimal sensitivity/specificity, but in most cases the parameter used for measuring the clinical usefulness of a panel developed by PLS, for example, is the R^2 (multiple correlation coefficient). R^2 of the model and R^2 of its cross-validation can be used as part of the biomarker choice process, but they do not provide enough transparency and are not a readily interpretable indication of the clinical usefulness of an assumed model. The ROC (receiver operating characteristic) curve analysis is a standard method for describing and

assessing the performance of medical diagnostic tests with binary classification [152]. Nonetheless, it is not used in plant metabolomics studies.

ROC curves reflect the regularity with which the test produces true positives, true negatives, false positives and false negatives, which are collected into the proportion of actual positives that are correctly classified as positive (sensitivity) and the proportion of actual negatives that are correctly classified as negative (specificity). An example of ROC curves is shown in Fig. 8.

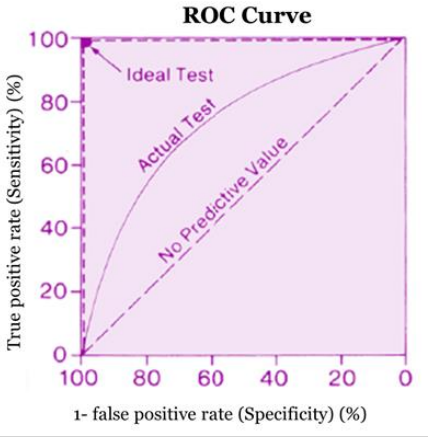

Fig. 8. ROC curve representation including the curve described for an ideal test, and a test without predictive value [153].

Specificity and sensitivity can be considered as the probability of false positives (the negative result of the test from a subject that has an actual negative outcome) and true positives (the positive result of the test from a subject that has an actual positive outcome), respectively. These parameters can fluctuate depending on the biomarker decision boundary, so the ROC curve is a good mode to visualize how sensitivity and specificity are affected by this decided threshold. ROC curve is considered an objective and statistically valid method for biomarker performance evaluation [152]; a no parametric measure of biomarker utility.

ROC curve generation and the area under the curve (AUC) —parameter that summarized the curve— can be generated by different informatics tools. AUC is interpreted as the probability that a diagnostic test or a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. If a perfect classifier is obtained, the

AUC value is 1, which mean that all positive samples are ranked before negatives ones. When AUC value is 0.5, the classifier does not present utility because it is equivalent to randomly classifying subjects as either positive or negative. Nevertheless, AUC may not be the most appropriate in some cases, where the partial AUC is more useful when only certain regions of the ROC space (high sensitivity or high specificity) are of particular interest [154].

3.4.2. Data treatment in targeted metabolomics analysis. The development of targeted metabolomics methods requires the optimization of experimental parameters for obtaining the best working conditions for the determination of the compound(s) of interest, through response surface or other designs [155,156]. The optimization designs can be classified into two main groups: i) univariate designs, in which a single factor is analyzed by the effect of its variation, while the rest of factors remain fixed. ii) multivariate designs, in which the interactions among different factors are studied [157]. Full factorial designs offer more information on interactions among factors, but the number of experiments multiplies substantially when many factors are analyzed. As example, in two-level full factorial designs for k factors (2^k), if k = 2, the required experiments are 4; if k = 4, the number of required experiments is 16; if k = 6, the number of experiments is 64, and so on. If more levels are analyzed, three level full factorial designs (3^k) must be implemented, if k = 2, the number of required experiments is 9; if k = 4, the number of experiments is 81, etc. If the effect of many factors has to be analyzed, the use of fractional factorial models decreases the number of experiments and provide information about the factors that exert strong effect on the response. In this way, the number of factors to be analyzed in successive experiments is reduced [158]. Among the existing optimization methods, response surface is the most common, although the simplex method or the sequential method, where each new experiment is based on the results obtained in previous experiments, are used in some cases [159].

Concerning calibration curves and characterization of a method, simple statisticals such as regression or calculation is used to obtain the calibration curves for quantitation of the target metabolites and to characterize the analytical method (reproducibility and repeatability, among others) in target analysis. Then, after integration of the target metabolite area, pre-processing strategies as normalization and/or scaling can be applied. The objective of the former strategy is to remove the unwanted systematic bias in ion intensities among measurements, while keeping biological variation. In scaling, each

variable is divided by a different factor to treat all variables equally, regardless of their intensity.

3.5. Identification of metabolites

The identification of the key metabolites is a requirement in untargeted metabolomics analysis, because it is needed for biological or chemical interpretation of the results of metabolomics analysis. Owing to the chemical diversity of most metabolomes and the character of most metabolomics data, metabolites identification is intrinsically difficult.

Identification of metabolites by MS requires a combination of high-resolution, accurate mass matching, MS/MS fragmentation data, and retention time comparisons between experimental data and pure standards or database, and libraries information [160]. When database searching results in multiple potential identifications for a particular mass, tandem mass spectra collected for each metabolite of interest can be compared to metabolite standards, libraries, or *in silico* fragmentation tools such as MetFrag for further narrow down identification assignments [161].

Pure standards of many compounds are not available, and also the cost of setting up large collections of standards is prohibitive. Publication and commercialization of electronic libraries from metabolomic analyses carried out by by GC–MS or LC–MS platforms reduce or minimize the problem. Retention times and characteristic ions of huge amounts of metabolites are listed in these libraries, each of which is specific to one type of column in LC, and to one type of derivatization and ionization (usually methoximation + trimethylsilylation and electron ionization) in the case of GC–MS libraries. The detected and no identified metabolites appear listed, together with their retention times and ion spectra as “mass spectral tags” or “events”.

Some of the most used metabolites databases with identification purposes are METLIN [162], HMDB (Human Metabolome Database) [163], PubChem [164], MassBank [165], MMCD (Madison Metabolomics Consortium Database) [166], and LIPID MAPS [167], as the most important. Many plant metabolites, mainly secondary metabolites, are not present in these databases; therefore, given researchers create their own and specific databases for plant metabolomics studies. Some researchers on plant metabolomics have developed specific databases and software that allow integration of data from different omics. This is the case of the Sumner group, who has reported a tandem mass spectral

library of plant natural products by using pure standards and purified compounds [168]. The database involves plant secondary metabolites, with their retention times and tandem mass spectra obtained at six collision energies.

3.6. Metabolic pathways interpretation

After a set of metabolites of interest has been identified and quantified, two tools can be used to gain biological insight into the experimental results: (i) mapping and visualization of pathways, and (ii) statistical enrichment analysis of metabolite annotations.

A first necessary step is the analysis of the metabolites set, the identification and interpretation of the metabolite data searching for the underlying biochemical mechanisms and their influence on phenotype and physiology of the target subject [169]. This means that the results provided by the applied metabolomics strategy need a secondary analysis to answer the initial question. A post-process of the metabolomics data is required for their biological interpretation (*e.g.*, pathways or biological processes overrepresented in these genes), which can consist of extracting recurrent patterns or enriched 'features' such as metabolic pathways, physico-chemical features, associated diseases, etc., from the list of metabolites identified in the experiment. For large-scale experiments involving a huge number of compounds these features can be extracted by sophisticated and automatic tools.

The software tools available for the biological and functional interpretation of metabolomics data are classified into two groups for complementary analysis. The first involves tools for mapping and visualization of a set of metabolites, thus allowing obtaining graphical plots of the metabolism, as metabolic pathways. The second group involves tools for the statistical analysis of metabolite annotations (enrichment analysis), and tools to perform metabolic modeling (or metabolic simulation).

3.6.1. Pathway mapping and visualization. The interpretation of metabolic experiments requires to map and visualize the identified metabolites and associate them to metabolic pathways and other biological networks. The visualization allows that the identified metabolites can be considered in their metabolic context, assessing whether them are involved in a common biological pathway or are related in a given metabolic network. The complexity of the metabolism and the associated biological phenomena require automated

and interactive tools to locate and visualize a number of compounds in metabolic charts. Most of the existing tools are not specifically designed for metabolomics analysis but they can provide information on the location in the metabolism of a given set of metabolites. They have been classified in two groups: those provided by pathway databases [BioCyc-Omics Viewer (http://biocyc.org), KEGG (http://www.genome.jp/kegg/pathway.html) and Reactome (http://www.reactome.org)]; and those developed by third-parties (independent laboratories) [iPath (http://pathways.embl.de), VANTED (http://vanted.ipk-gatersleben.de), Metscape (http://metscape.ncibi.org)].

Other tools are focused on the integrated visualization of omics data such as ProMetra [170] (a web-based multi-omics pathway mapping and visualization tool), Paintomics [171] (a web-based tool that maps simultaneously quantitative data from both, transcriptomics or proteomics and metabolomics experiments in KEGG pathways), and Pathvisio, an application for the creation and edition of pathways, can also be used to map experimental data associated to both, gene/proteins and metabolites. There are two tools of interest to study plant metabolomes: KaPPA-View [172] and MapMan [173]. Other pathway analysis and visualization tools constitute a part of metabolomics data analysis software such as MetPA [174] (integrated in the MetaboAnalyst platform [175]), and Pathos [176] (integrated with MeltDB [177]).

3.6.2. Enrichment analysis. Statistical values associated to gene functional annotations can be provided by several tools applied for enrichment analysis to transcriptomic and proteomic data. The annotations in a set of given genes can be compared with the corresponding annotations in a reference set, and those overrepresented in the given set be reported, according to a given statistical test. Then, the tools are used to 'summarize' a list of genes and elucidate the biological phenomenon behind it. Available software implementing similar approaches for the analysis of a list of metabolites are MSEA (http://www.msea.ca), MBRole (http://csbg.cnb.csic.es/mbrole), MPEA (http://ekhidna.biocenter.helsinki.fi/poxo/mpea/) and IMPaLA (http://impala.molgen.mpg.de). In this way, functional interpretation of metabolomic experiments on statistically significant pathways and on other biological annotations can be achieved.

Types of analysis. Overrepresentation analysis (ORA) and set enrichment analysis (SEA) are two tools for enrichment analysis.

ORA allows development of a statistical test to check if a set of metabolites is enriched with a particular annotation as compared to a background set. The statistical test is applied to each annotation of the target set of metabolites for calculation of a *p*-value. ORA manages a given metabolite set and provided information on its underlined biological mechanisms and functional implications. Chi-square, Fisher's exact test, binomial probability and hypergeometric distribution are some of the statistical tests used for analysis [178]).

SEA uses a list of genes in the array sorted by a score (*i.e.*, fold change of expression) to define a list of overexpressed or underexpressed genes, looking for annotations to be associated to extreme scores in a sort of 'continuous' way [179]. In its application the analysis of metabolite sets is performed by associating a quantitative measure to the concentration of each metabolite. The consistency of each annotation in the top and bottom of the list of metabolites sorted is evaluated by that quantitative value, which compared to a background distribution.

3.7. Validation

The correct biological interpretation of a specific metabolite difference in the untargeted approach depends on the reliability and suitability of the whole approach (from sampling to biomarker identification) [180]. Obtainment of consistent and reliable data from the analytical measurements is the key to avoid false positive and negative values. It means that validation of the analytical methods is needed to achieve the objective.

For targeted metabolomics, validation of available guidelines is specifically focused on instrumental aspects. There are no guidelines available for untargeted metabolomics, so alternative ways to validate untargeted approaches are being used. Some available guidelines exist for analytical and bioanalytical aspects as the two industrial guidelines established by the United States Food and Drug Administration (FDA) [181,182], other two guidelines develop by ICH for method validation that were later merged in one (Q2-R1) [183], and one published by the International Union of Pure and Applied Chemistry, called "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis". This last guideline provides scant recommendations on procedures that should be used to confirm validation of analytical methods [184].

All these guidelines are mainly focused on seven (accuracy, precision, specificity, LOD, LOQ, linearity and range) parameters that should be controlled during validation of a bioanalytical method to get the searched purpose.

3.7.1. Untargeted metabolomics and method validation. Guidelines for validating the analytical part in untargeted approaches do not exist. Most of the times the purpose of untargeted metabolomics is to discover statistically significant biomarkers doing unbiased differential analysis of the biggest amount of signals from biological samples, which will compare different conditions under study. Comparisons will be valid when the samples are studied under the same conditions and the method and the signal variation is related to the metabolites concentration. So, it is important to develop a method that includes all necessary steps prior to validation.

Some alternative strategies to validate the analytical method have been used and are described below.

Quality control samples (QCs). Some potential limitations should be controlled while developing and applying high-throughput techniques for the untargeted approach to eliminate bias owing to a gradual change in the performance of the system. These limitations can yield potential drifts in chromatographic and MS performance, thus affecting the repeatability and reproducibility of the method. To monitor the performance of the instruments, the order of the samples must randomized, and quality QCs should be included [185].

QCs are pools of samples prepared either from the target test samples, or a representative bulk control sample, and they should be assessed against preset criteria by which acceptance or rejection of the analytical run is decided. A number of injections should be made at the beginning and end of the analytical run, and repeated at regular intervals throughout the sequence run, with the aim of stabilizing the system. Other criterion suggested by the FDA for validation of bioanalytical methods consists of calculation of the relative standard deviation (RSD) of the molecular features existing in the QCs [186]. Values close to 30% RSD could be allowed for untargeted metabolomics analysis by LC–MS. Together with checking RSD in QCs, the linearity of diluted pools of QCs, matrix effects and recovery have been checked by some researchers [187].

Spiked standards. The use of the external standard method is frequent in the pharmaceutical industry to control the reliability of the methodology. This kind of

validation has been used by some researchers in untargeted analysis. As example, six xenobiotic standards were spiked to calculate the matrix effect and recovery in the validation of a quantitative method applied in nutritional plasma metabolomics [188]. Although spiking some chosen metabolites provides an approach to the general behavior of the method, the behavior cannot be supposed for all the compounds in the sample. Internal standards have also been used to check instrumental reproducibility [189].

Also conventional validation guidelines have been applied in some cases; nevertheless, not all them can be used for method validation. However, to target some of them from no targeted metabolites (considering different class/category, retention time/migration time, more or less abundant, some physico-chemical properties) should be acceptable to validate some methodologies.

Statistical model validation. Either targeted or untargeted metabolomics analysis uses the tight clustering of QCs in the PCA model to check the quality of the instrumental platforms. By the poor or good clustering of QCs, an initial idea of the data could be got, and before making a PCA model the QCs can be excluded and later predicted on the same model. The clustering of the QCs after prediction considers the repeatability of the instrumental performances and established that the separations between the groups are not due to the instrumental variations but to the sample itself [190].

3.7.2 Chemometrics model validation. After validation of an analytical method, the chemometrics models must be validated. The prediction reliability is of primary concern in the evaluation of the performance of a given regression model.

External validation is referred to as the validation of models by objects (samples), whose data were not used for model calculation. Objects excluded from the model fitting are known external test objects, and they should not be confused with test objects used for the internal validation of models. The main difference between the two sets is that the former is used only for validation purposes, while the latter also used for model calculation and optimization. External test objects should be arbitrarily selected from the pool of existing objects and they must be fully independent of the training set.

Strategies based on cluster analysis, optimal design or sphere exclusion algorithms are applied to design an external test set that results enough representative of the whole set of data [191]. In this way, the test data lose their status of independence of training data.

4. Recent contributions of the FQM-227 research group to plant and clinical metabolomics

The group in which the PhD student has developed her PhD is "Analytical platforms in metabolomics: clinical and agrifood areas", FQM-227, as a member of the PAI, and therefore of the University of Córdoba; and "GC-21 Metabolomics" as a member of the IMIBIC. The main research lines of this group are metabolomics (including lipidomics, nutritional metabolomics, search for clinical metabolomics biomarkers, plant metabolomics, and analytical omics platforms based on mass spectrometry), analytical green chemistry, exploitation of agrifood byproducts, automation and acceleration of sample preparation by microwaves, ultrasound or superheated liquids assistance. The wide experience of the group in the development, validation and application of metabolomics-based analytical platforms is remarkable.

Clinical metabolomics studies developed by the group in the last five years include a variety of samples, from no invasive and scarcely used human biofluids as sweat [192] or exhaled breath condensate [193] to common biofluids as urine [194] or blood [81]. All kind of research, from optimization studies [195] to creation of data treatment tools, has so far been developed by the group [196]. Among others, the group has developed optimization studies for metabolomics untargeted analysis of human sweat and exhaled breath condensate by LC–MS/MS [195] and GC–MS, respectively, both in high resolution mode [197]. After characterization of the target samples, discrimination of different groups of samples has been achieved. Thus, sweat has been the biosample involved in metabolomics studies for lung cancer screening [192]; exhaled breath condensate has been the sample for discrimination of either individuals with different smoking habits by GC–TOF/MS [198] or lung cancer patients from risk factor individuals [199]; and also for identification of metabolomics panels with potential for lung cancer screening [193]. Urine has allowed discriminating prostate cancer patients and negative biopsy controls [194].

Other clinical metabolomics studies have been focused on the complex pathways in which vitamin D is involved. After development of analytical methods to determine in 200 μL of serum samples up to 8 vitamin D metabolites [82] —including the C3-epimer-25-monohydroxyvitamin D_3, which has required the use of two-dimensional LC coupled to an MS/MS detector [200]— the method thus developed has been massively applied to serum samples from cohorts constituted by breast cancer patients, and premenopausal women, among others [201,137]. The results from application of the method to massive

number of samples have allowed finding out the relation between 25-hydroxyvitamin D in serum and mammographic density in premenopausal Spanish women [137], among other findings. Other interesting recent studies developed by the group have involved endocannabinoids and analogous compounds, for which a method based on LC–MS/MS in MRM mode, and on a key serum-sample preparation step has been reported [202]; and the quantitative determination and confirmatory analysis of N-acetylneuraminic and N-glycolylneuraminic acids in serum and urine by SPE on-line coupled to LC–MS/MS [203].

Regarding plant metabolomics, methods for sample preparation and analysis have been developed for a great variety of samples, involving traditional samples such as olive leaves and fruits, wine and vineyard material, but also samples of recent interest in the agrifood area both fruits and byproducts and also waste of them. Examples of these studies are the characterization of polar and no polar compounds in stevia leaves [204], wine lees [205] or Persian lime (*Citrus latifolia*) [50], and sample preparation for quantitation of families of compounds such as flavonoids in orange peel [206]. Characteristic of the group are the studies on the effect of auxiliary energies on the extraction of plant metabolites [207] or that of evolution of the composition of polar or no polar fractions components of a fruit during the grown period, performed by both LC–QTOF MS/MS and GC–TOF/MS platforms [49,207]. Other studies have been focused on compounds with healthy effects, as is the case with oleocanthal and oleacein in virgin olive oils, which were quantified by LC–MS/MS [208]. Also, the effect of these compounds in humans and animals has been considered, such as the decrease of the postprandial inflammatory response by reducing postprandial plasma lipopolysaccharide levels in metabolomic syndrome patients by olive oil phenolic compounds [209] or the inhibitory effects on liver carcinogenesis in rats by red and white wine lees [210]. Furthermore, the group has elucidated the metabolism of some pesticides such as imazamox in common plant and in plants with genetically modified resistance, such as Clearfield® wheat [211].

Finally, the group have also experience in acquisition and treatment of metabolomics data, as shown in the following publications: MSCombine: a tool for merging untargeted metabolomic data from high-resolution mass spectrometry in the positive and negative ionization modes [196], and in enhancing metabolomics detection and identification by LC–MS/MS untargeted analysis in combination with gas-phase fractionation [212].

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Herramientas y equipos analíticos

Analytical tools and equipment

This section of the Thesis-Book briefly describes the different types of samples that have been the object of the research, the instruments and devices used during the experimental development of the Thesis, as well as the chemometrics tools and databases. In Chapters IV-to-XI a more detailed explanation of those that have been used in the research collected in each of them is included.

1. Samples

For the development of the research contained in Section B, three types of plant samples have been used; residues from the olive oil industry, such as olive (*Olea europaea* L.) leaves (Chapter IV), white button mushrooms (*Agaricus bisporus*) (Chapter V) and *Cannabis sativa* L. leaves and inflorescences (Chapter VI).

For Sections C and D, only a type of biological samples has been used: human sweat (Chapters VII-to-XI).

2. Sample preparation devices

Discontinuous equipment for sample preparation has been used in both targeted and untargeted analysis. In general, sample preparation was based on agitation, centrifugation, dilution, extraction, precipitation of proteins and/or derivatization. The required steps depended on the metabolites of interest, sample nature and chromatographic separation (GC or LC) used.

In plant metabolomics, obtaining of plant extracts required drying, chopping or grinding of the leaves, and subsequent extraction with a solvent or a mixture of them (see Chapters IV and VI). Methanol and water–methanol mixtures were the solvents used for polar compounds extraction, and hexane for no polar compounds. The extraction step was assisted by agitation (using a Vibromatic rocking mixer) (Chapter IV) or US (using an US bath at 37 kHz and 800 W) (Chapter VI).

Mushroom extracts required homogenization, enzymatic digestion, centrifugation of the hydrolyzate and collection of the supernatant (mushroom extract) (see Chapter V). Fractionation of the extract required LLE with different polarity index extractants. In

Chapter IV, the olive leaf extract was subjected to US-assisted enzymatic hydrolysis (USAEH) of oleuropein to its aglycon, which required controlled conditions of enzyme amount, buffer pH, temperature (by a digital block heater) and optimized US conditions (US cycles, duty cycle, % amplitude and cycle time), for which an ultrasonic probe (20 kHz, 400 W), equipped with a cylindrical titanium alloy probe microtip was used.

In clinical metabolomics, simple protocols as dilution with an acid (Chapter XI) or dilution+clean-up by c-μSPE (Chapter VII) were used. In other cases, focused to coverage the sweat metabolome, protocols as deproteination (Chapter X) and LLE with different extractants (Chapter VIII) were used. Deproteination+acidification was used in Chapter IX.

In the research involving GC, the sample preparation protocol required removal of water traces (dryness or freezing) and derivatization of the extract (using a ThermoMixer C to heat and mix). Methoxymation+silylation (Chapter VIII-to-X) or only silylation (Chapter VI) were the derivatization protocols used.

3. Detectors and previous chromatographic separation systems

The methods developed in the experimental part of this Thesis have been based on chromatographic separation by LC or GC, and subsequent detection by mass spectrometry.

For untargeted analysis to obtain the metabolomics profile of plant extracts (Chapters V and VI) and human sweat (Chapters IX and XI), an Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with dual electrospray ionization (ESI) was used, with detection in MS/MS mode. In all cases, the separation of the metabolites was based on C_{18} reverse-phase analytical columns (25 cm×0.46 cm i.d., 5 μm particle size in Chapter V; 25 cm×0.46 cm i.d., 3 μm particle size in Chapter VI; and 5 cm×0.46 cm i.d., 3 μm particle size in Chapters IX and XI), using water and methanol (Chapter V) or acetonitrile as mobile phases, and formic acid as ionization agent.

Targeted analysis, used in Chapter IV and VII, was performed by an Agilent 1200 Series LC system (consisting of a binary pump, a vacuum degasser, an autosampler and thermostated column compartment), and an Agilent 6460 triple quadrupole mass spectrometer equipped with a Jetstream® electrospray ionization (ESI). Chromatographic

separation was performed by C_{18} reverse-phase analytical columns (15 cm×0.46 cm i.d., 5 μm particle size in Chapter IX; and 10 cm×0.46 cm i.d. 3 μm particle size in Chapter VII), using water and methanol or acetonitrile as mobile phases, and formic acid or ammonium formate as ionization agent. In all cases, the data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis.

Untargeted analyses of volatiles or easy to convert into volatile compounds were performed using always the same plaftform (Chapters VI and VIII-to-X): an Agilent 7890B Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with electron impact source, using a fused silica DB-5MS-UI 30 m×0.25 mm i.d,×0.25 μm film thickness capillary column from Agilent Technologies.

4. Chemometrics tools

According to the importance that chemometrics has acquired in metabolomics, in the research included in this Thesis-Book, chemometrics tools have been used both for the development and optimization of analytical methods and for the treatment of multivariate data. First of all, data processing was carried out with different software according to the objective and instrument used:

i) Extraction and alignment of molecular entities in untargeted analysis provided by an LC–QTOF platform. A combination of Agilent softwares was used: Qualitative Workstation+Profinder or MassProfiler Professional. The first software allows extracting the entities taking into account adducts and isotopes, the second and third allow alignment of the potential entities.

The Qualitative Workstation software was used to process the data obtained by LC–QTOF in auto MS/MS mode. Treatment of raw data files started by extraction of potential molecular features with the suited algorithm included in the software. Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities and other contaminants after analysis of a blank of each sample. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention times, intensity in the apex of the chromatographic peaks and accurate mass (Chapters V, VI, IX and XI).

The "Recursive Feature Extraction" algorithm from the Profinder software was used to extract and align potential molecular features in all injections. This algorithm initially deconvolutes chromatograms and aligns features across the selected sample files in terms of mass and retention time; then, it uses the mass and retention time of each feature for recursive targeted feature extraction. This two-step procedure reduces the number of both false negatives and false positives in feature extraction (Chapter XI).

The "Batch Targeted Feature Extraction" algorithm from the Profinder software was used to extract and align peaks from all chromatograms that matched a database of the identified metabolites (Chapters VI and IX).

The MassProfiler Professional was also used to align potential molecular features in all injections (Chapter V).

ii) Extraction and alignment of molecular entities in untargeted analysis using the GC–TOF platform. The combination of three Agilent softwares was used: Unknown Analysis, Qualitative Workstation and Quantitative Workstation. The first allows unzipping all data files obtained by GC–TOF/MS in full scan mode; the second allows extracting the potential molecular entities from an untargeted analysis; the third software is used to re-extract the entities in all samples (recursive analysis).

The Qualitative Workstation software was used to process all data obtained by GC–TOF/MS in full scan mode. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of molecular features (MFs) considered as potential compounds defined by the *m/z* value and retention time (RT) of one representative ion for each chromatographic peak. The background spectrum was subtracted to the spectrum obtained from each sample before its deconvolution (Chapters VI and VIII-to-X).

For recursive analysis, the Quantitative Analysis software was used to reintegrate all potential compounds found in all analyzed samples, using the corresponding characteristic quantifier and qualifiers (Chapters VI, VIII and IX).

Once data pre-processing has finished, the data pre-treatment step (which consists of applying mathematical operations to favor comparison among samples) starts.

Normalization of the data was performed by the Mass Profiler Professional, before the statistical analysis (Chapter VIII), and also by MetaboAnalyst (www.metaboanalyst.ca). The latter was used for normalization by sum (the quantitative response of a potential

metabolite is scaled by the sum of the quantitative responses of all potential metabolites detected in the samples), log transformation and scaling of the data set (Chapter XI).

Regarding types of filters, filter by frequency was required in Chapter V. The Mass Profiler Professional software was used to reduce the molecular features number, based on frequency of occurrence by comparing repetitions of the same sample.

Among the statistical analysis tools and softwares used in the studies of this Thesis-Books the following are remarkable:

- Statgraphics: software that allows performing different univariate and multivariate statistical analyses, as well as the design and evaluation of screening models and response surfaces. It was used for optimization of experimental parameters to obtain the best working conditions for the determination of the compound(s) of interest, through response surface. A multivariate design, a 15-run BBD, was used to optimize three interrelated US variables: X1 (duty cycle), X2 (amplitude) and X3 (cycle time) for the enzymatic hydrolysis of oleuropein (Chapter IV). Other multivariate design involving 3-level 32 factorial designs including 9 experiments and 2 central points, which corresponded to star points used to model the surface, was used to evaluate the nozzle voltage and sheath temperature of the source for amino acids quantitation (Chapter VII).

- MassProfiler Professional: software that allows application of different statistical analysis algorithms especially suitable for metabolomics analysis. It was used for statistical analyses in Chapters IV and VIII.

- MetaboAnalyst: web tool that allows executing high performance analytical studies in metabolomics for the analysis and interpretation of metabolomics data. MetaboAnalyst was used for metabolomic data analysis, visualization and interpretation in Chapters VII and XI.

- SPSS Statistics: software package used for interactive or batched statistical analysis. This base software includes descriptive statistics (cross tabulation, frequencies, descriptives, explore, descriptive ratio statistics), bivariate statistics (means, *t*-test, ANOVA, correlation, no parametric tests, Bayesian), linear regression and prediction to identify groups (factor analysis, cluster analysis and discriminant analysis). SPSS was used to performed the statistical analyses in Chapters V and IX, and PASW Statistics was used to statistically analyze the differences in the content of cannabinoids and terpenoids among different varieties of cultivars and cultivation modes (Chapter VI).

The evaluation of the predictability of metabolites or panels with potential as markers has been carried out through Random Forest graphs and ROC curves, which represent specificity versus sensitivity. The free online access tools ROCCET (http://www.roccet.ca/ROCCET/) and Metaboanalyst have been used to obtain them. In Chapter XI, the predictive capability of each metabolite by Random Forest Analysis was determined by MetaboAnalyst.

Design of prediction models and creation of marker panels: in this case we have used the online tools ROCCET, MetaboAnalyst and the PanelomiX software, which allow creating marker panels and assess their prediction/discrimination ability. In Chapter XI, PanelomiX toolbox, supported on the iterative combination of biomarkers and thresholds, was used to combine iteratively biomarkers by selecting thresholds that provided optimal classification performance [1].

5. Identification of metabolites and databases

Identification of the most relevant entities was supported on MS and MS/MS information, which was obtained by LC–QTOF MS/MS and analyzed using the MassHunter Qualitative Data Analysis v.7.0 program (Agilent Technologies, Inc. 2014), by comparison of the MS/MS spectra obtained with those stored in libraries generated by the research group [2], as well as in the public databases such as METLIN MS and MS/MS databases (http://metlin.scripps.edu); the Human Metabolome Database (HMDB) (http://www.hmdb.ca) and the Metfrag database (https://msbi.ipb-halle.de/MetFrag/) based on mass spectra of metabolites generated by *in silico* fragmentation (Chapters V, VI, IX and XI). In addition, standard solutions were used to confirm the presence of identified compounds (Chapter VI).

The identification of the metabolites detected by GC–MS was firstly carried out by searching MS spectra in the NIST Mass Spectral Search v.11.0 (NIST, Washington, DC, USA). Only identifications with a match factor and a reverse match factor higher than 700 were considered. Furthermore, the RI values included in the NIST database were used to support identifications. For this purpose, an RI calibration model was built by plotting the retention times (RT) obtained by analysis of the alkane standard mixture (C10 to C40 with an even number of carbons) with the chromatographic method used in this research and the RI values provided for each alkane by the NIST database. This equation was used to

estimate the RI value for each identified compound, which has to be less than 100 units different as compared with the theoretical value provided by the NIST. This database does not contain high resolution MS information as provided by the TOF detector. For this reason, a third step was included to validate identification of each compound by using high resolution MS. Thus, the molecular formula for the tentative ion $[M]^+$ and the most intense fragments for each molecular feature (MF) should fit the NIST identification by setting a cut-off value in mass accuracy of 10 ppm (Chapters VI and VIII-to-X).

Identification was also carried out by searching MS spectra on the Fiehn database [3]. Only identifications with a score higher than 70 were considered as valid. The RT included in the Fiehn database were also taken into account to support identifications with the requirement for acceptance of a difference between the experimental RT and the theoretical value provided by this database for each target compound below 0.50 min (Chapters IX and X).

Additionally, multistandard solutions of terpenoids and cannabinoids were injected to confirm their identification in the *Cannabis* extracts (Chapter VI), and derivatized standards (glutamic acid, phenylalanine, proline, D-galactose, D-glucose, myo-inositol, phosphate, *p*-hydroxybenzoic, fumaric, malic, succinic, caprylic, lauric and palmitic acids) were injected for confirmation of some amino acids, sugars, benzene derivatives, dicarboxylic and fatty acids in sweat (Chapters VIII-to-X).

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Parte Experimental

Experimental part

Estudios bibliográficos

Bibliographical studies

Scientist training requires, as a starting step, an in depth study of the literature related to the planned research; then, a continuous updating during the development of target studies. The previous study of the related literature allows establishing the limits between what is done and what should be done to advance in or start a given research line. The continuous update is the way to keep the research in the front line.

An added value to an in depth study of the bibliography on a certain topic can be obtained if the knowledge acquired is sufficient to be offered to potential readers in the form of either a revision or a book chapter. This has been the case with the training of the PhD student on sample preparation, one of the research guidelines of the team to which she belongs, and in which the use of ultrasound (US) is a hallmark.

In this way, the effect of US on the extraction of food components has given place to a huge number of methods that clearly surpass the analytical features of conventional methods and even those of methods assisted by other energies, such as microwaves. Chapter I of this PhD book is in turn a chapter belonging to an online Reference Module in Food Sciences, published by Elsevier, in which typical and special US-assisted methods for extraction of food components are explained and discussed; then compared with other methods using different energy assistance or even dual energy assistance.

Chapter II constituted a new step in the literature training of the PhD student as it is devoted to the use of US for sample preparation in the metabolomics field. Thus, the published review critically discusses the most abundant uses of US in the plant area to the detriment of the clinical area. Special emphasis is devoted to the almost total absence of studies on the potential degradation of given sample components caused by US, as only the efficiency of the favored step is outlined without taken into account the degradation that US energy can promote on key and/or unimportant compounds.

The very controversial information in the bibliography related to the influence of US on biocatalysis has been the subject of an also controversial review that constitutes Chapter III. The scan attention paid to the frequency and power of the US device used, and

the absence of crucial data and metadata in most publications hinders to draw conclusions from the study of the existing bibliography. The assessment of this situation gave place to the development of the research in Chapter IV (Section B) as a starting point of the future wide investigation required to give enough light on the US–enzyme binomial. Rational use of US, either to accelerate or hinder enzymatic activity, will be the most useful result of these studies.

Chapter 1

Ultrasound-assisted extraction of food components

Ultrasound-assisted extraction of food components

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Chapter in: Reference Module in Food Science, 2017.

Ultrasound-assisted extraction of food components

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Synopsis

The growing use of ultrasound-assisted extraction (USAE) with foodstuffs is discussed. The chapter starts with a general overview on the ultrasound (US) basics and the features that assist in extraction, the extractants and US devices used. Representative examples of USAE methods used for the extraction of food components (*e.g.*, antioxidants, volatiles, multielements, undesirable compounds) are critically discussed. Further USAE methods for foods based on the use of ionic liquids (ILs), a typical US property of favoring emulsification simultaneously with extraction —both involving not only solids but also liquid samples—, development of USAE with either simultaneous derivatization or enzymatic catalysis are discussed. A comparison of USAE methods with other conventional or unconventional extraction methods with respect to the respective advantages and disadvantages is also covered. The exploitation of USAE at pilot scale and industrial scale is discussed with a view to a wide use of this technology at a scale greater than laboratory scale. Finally, the foreseeable and desirable future trends are considered.

1. Introduction

Ultrasound (US), or sound of frequencies above the capabilities of human hearing, spans within the range from 20 kHz to several GHz, which can be divided into a power region and a diagnostic region. The low-frequency region is endowed with enough ultrasonic energy to produce cavitation, which is of great interest from the analytical point of view for sample preparation and from the industrial point of view to produce key effects on given steps of the production line. The cavitation phenomenon is highly dependent on the US frequency (a property that should be taken into account by the users), and it consists of three main components: thermal effects, shear forces, and free radical formation (the last only when applied in polar liquids).

1.1. General aspects of ultrasound-assisted extraction

The favorable effect of US on mass transfer between two phases in the same or different physical state has been widely demonstrated as did the number of chemical reactions accelerated/improved under ultrasonic influence (Luque de Castro and Priego-Capote, 2006). Thus, US application allows to save energy, and also to be operated efficiently at moderate temperatures, which is beneficial for heat-sensitive compounds. When applied to the removal of given components from a solid sample (known as solid–liquid extraction, leaching, or lixiviation), US gives place to phenomena that affect the raw material through effects such as fragmentation, erosion, detexturation, and also to an effect referred to as ultrasonocapillary effect or increase of depth and velocity of penetration of the extractant into channels and pores under given US conditions (Chemat *et al.*, 2017). In dealing with plant cells or tissues, the US effect involves cell membrane destruction, thus causing morphological changes of the plant structure that can change its properties dramatically. An example of this behavior can be seen in Fig. 1, which shows photomicrographs of saffron before and after ultrasonication (Sereshti *et al.*, 2014). The effect is enhanced by the simultaneous action of other types of treatments, as that provided by extractants under supercritical (SC) conditions. This is the case with the supercritical fluid extraction (SFE) with SC carbon dioxide of bioactive components from blackberry (*Rubus sp.*) industrial residues, which was enhanced by US application. Scanning electron microscopy (SEM) was used to analyze the blackberry bagasse subjected to SFE with and without US assistance, which showed that this treatment disturbs the cell walls, enhancing

the release of the extractable compounds. This can be seen in Fig. 2 showing the effect of SFE and USAE separately and combined (Pasquel Reátegui *et al.*, 2014). These studies are of interest to explain the increased extraction efficiency (of phenols and anthocyanins in this case) when these types of materials are subjected to the combined treatment, and also to understand, from an economical viewpoint, the final state of the residue after extraction.

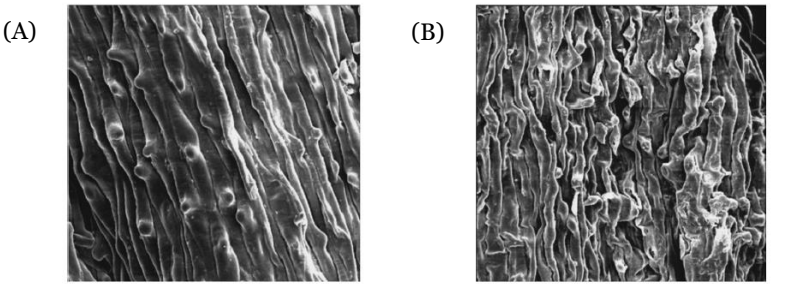

Fig. 1. Morphological photomicrograph of saffron: **(A)** before ultrasonication and **(B)** after ultrasonication. With permission of Elsevier, Sereshti, H., Heidari, R., Samadi, S., 2014. Determination of volatile components of saffron by optimized ultrasound-assisted extraction in tandem with dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry. Food Chem. 143, 499–505.

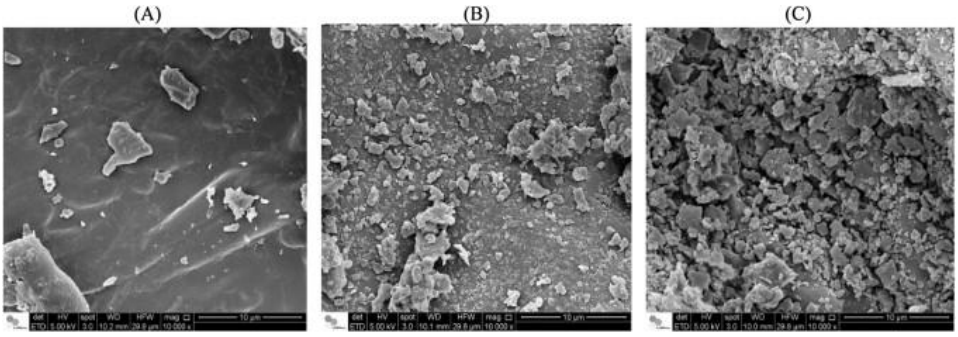

Fig. 2. Images obtained by SEM on the surface of blackberry bagasse particles under different conditions of extraction: **(A)** SFE without US at 50 ºC and 25 MPa; **(B)** SFE + US at 40 ºC, 15 MPa and a 200 W US power; **(C)** SFE + US at 50 ºC, 25 MPa and a 400 W US power. *SEM*, scanning electron microscopy; *SFE*, supercritical fluid extraction; *US*, ultrasound. With permission of Elsevier, Pasquel Reátegui, J.L., da Fonseca Machado, A.P., Barbero, G.F., Rezende, C.A., Martínez, J., 2014. Extraction of antioxidant compounds from blackberry (*Rubus sp.*) bagasse using supercritical CO2 assisted by ultrasound. J. Supercrit. Fluids 94, 223–233.

The lower operating temperature in USAE, as compared to microwave-assisted extraction (MAE), does not avoid the degradation effect of free radicals formed by polar extractants. While USAE can be a suitable technique for the extraction of thermally unstable compounds, one should not forget the potential effect of free radicals, which mainly affect to the compounds extracted at long US application times. The degradation effect is shown as a decrease of the extraction yield when increasing the extraction time if the process is monitored by an unselective detector (Sun *et al.*, 2011).

1.2. Extractants in ultrasound-assisted extraction

The extractant features can influence USAE efficiency drastically, and its effect is highly dependent on the nature of both the matrix and analyte(s). Fig. 3 shows this effect on the extraction of phenols from pomegranate peel using extractants of different polarity. The behavior shown in the figure was expected, given the polar nature of the extracted components (Kaderides *et al.*, 2015).

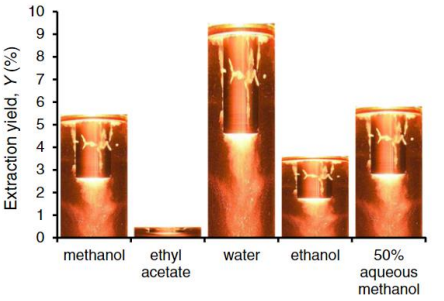

Fig. 3. Effect of the extractant on the extraction yield of phenols from pomegranate. With permission of Elsevier, Kaderides, K., Goula, A.M., Adamopoulos, K.G., 2015. A process for turning pomegranate peels into a valuable food ingredient using ultrasound-assisted extraction and encapsulation. Innov. Food Sci. Emerg. Technol. 31, 204–215.

The present trend of promoting green extractants has led to the use of oils for extraction. However, oils as extractants have a limited application as they can only be used to be enriched with the components from the solid that are able to be transferred to the lipids. Isolation of the transferred compounds is a very difficult task. Recently, USAE has

been used to transfer carotenoids from pomegranate to sunflower and soy oils (Goula *et al.*, 2017), thus improving the antioxidant capacity of the oils. Nevertheless, when dealing with oils, it is important to consider that US can drastically affect oil stability. This behavior was demonstrated by the authors in a study on oils' stability in which stable extra-virgin olive oil showed severe rancidity after only a few hours of US application (Cañizares *et al.*, 2004).

The growing trend to the use of multivariate optimization designs (*e.g.*, central composite rotary design, faced-centered cubic experimental design, completely randomized two-factorial design, Box–Behnken design), which reduces the number of experiments to be carried out in the optimization process, hides the behavior of each individual component when its value is changed. This negative aspect of multivariate optimization is absent in univariate optimization, which should be applied when possible.

1.3. Ultrasound devices

There are currently two main types of US equipment available in the market for analytical laboratories (plus a further type, plate transducers, which is not to be discussed in this article): ultrasonic cleaning baths and probes. The former (designed mainly for cleaning glassware and degassing) is omnipresent in any laboratory, because it is the most affordable and inexpensive US device available in the market. However, US cleaning baths have several major disadvantages due to the design of these baths (with transducer elements embedded in the tank walls). Ultrasonication is generally highly no uniform, which leads to problems with reproducibility of effects and performance. Furthermore, the transferred power into the tank declines over time, mainly due to a degradation of the transducer elements. Very often, users do not consider these aspects that can severely affect the results. In addition, not all US cleaning baths operate at the same frequency; a fact that can significantly affect the results, which is obviously a big problem when reproduction of previously reported results is attempted.

US probes, also referred to as horns or sonotrodes, are not subjected to the disadvantages of ultrasonic cleaning baths. In addition, probes are endowed with the versatility of power and duty cycle programming as required, so they are better suited to facilitate analytical steps in laboratories than US cleaning baths. Besides, microtips can be coupled to the transducer to work at low-volume scale.

Sonoreactors are US devices that work in tank format, but are well designed to operate more uniformly and provide good reproducibility, provided sample location and setup are fixed; therefore, these are more appropriate for analytical purposes. A similar design to analytical sonoreactors is adopted for pilot- and industrial-scale reactors (Chemat *et al.*, 2017).

2. Typical ultrasound-assisted extraction of food components

USAE methods are often grouped according to some common characteristics of the food (*e.g.*, antioxidant capacity, volatility, designed for extraction of volatile compounds, or because some food contaminant requires to be determined). Their main characteristics are discussed below.

2.1. Extraction of antioxidant compounds and other families of valuable compounds from plants/foods

There is a growing trend to extract families of compounds with given characteristics from a raw material, which can be used as nutraceuticals or to enrich/supplement foods. The raw material can be a complete plant or a part of it (*e.g.*, leaves, fruits, flowers, roots), a low value residue or waste. US is often an excellent treatment to accelerate extraction, improve the yield, and facilitate automation of the process. A plethora of USAE methods has been developed with the aim to obtain valuable plant components.

In most of the published USAE methods the extracted compounds are not identified, but rather global parameters of the extract, such as the antioxidant activity, the radical scavenging activity, the reducing antioxidant power, the total phenols content, or the flavonoid content, are measured during optimization and as a final result of the method (Briones-Labarca *et al.*, 2015). In most cases, common photometers for monitoring the given property of the extract are reported; therefore, degradation of the extract components under US application is only detected by a decrease of the measured absorbance. However, identification of the extracted components is essential when the aim is to use the extracts in food products to ensure that the degradation products are not toxic. Recent examples on this field are discussed below.

The term “bioactive compounds” is very broad and covers, for example, those compounds extracted by Tomšik *et al.* from *Allium ursinum* using an ethanol–water mixture under US application and optimizing the process by response surface methodology (RSM) (Tomšik *et al.*, 2016). Photometric overall measurements of total phenols utilizing the Folin–Ciocalteu (F–C) method and gallic acid as general standard, flavonoids by the aluminum chloride colorimetric assay, and scavenging capacity by the DPPH radical scavenging assay were carried out, but none of the extracted compounds were identified. Numerous figures showed the extraction yield as a function of each of the two relevant variables, while a table of the ANOVA results highlighted the importance of solvent concentration, as the authors considered the concentration of ethanol in the extractant mixture.

A further example was given by Wang *et al.* (2016) in the research on blueberries, cherries, and red pear peels attempting to achieve a high recovery of phenols and anthocyanins. The authors determined in the extracts the overall phenol content (F–C method), total monomeric anthocyanin content (differential pH method), total polymeric anthocyanins through percent polymeric color, and DPPH scavenging activity. Then, the extracts were injected into a liquid chromatograph (LC) for separation of the components and monitored at 540 nm by a photometric detector. Three of the anthocyanins in the extract were quantified by commercial standards, while those with no available standards were identified by bibliographic information and semiquantified by standards close to their chemical structure. Comparison of conventional extraction and USAE showed that different operating conditions must be implemented for maximizing extraction yield and anthocyanins composition of the extracts from the different fruits.

An extraction method based on discontinuous application of US was investigated to achieve high extraction yield and antioxidant activity of the extract from pomegranate peel. The main aim was the extraction of ellagitannins, phenolic acids, and flavonoids, significantly concentrated in pomegranate peel, to which health contributing properties, such as anticarcinogenic, antimutagenic, antitumoral, antidiabetic, and antioxidant properties have been attributed (Kazemi *et al.*, 2016). Two commercial standards of punicalagin and ellagic acid were used for identification and quantitation of these compounds, while other extract components were identified/semiquantified by bibliographic data. Enhanced extraction yield and shortening of the time were achieved

under the optimum conditions of the USAE method, considered by the authors as practical for large-scale industrial food production.

In dealing with USAE of valuable compounds in orange peel, a method was developed in 2010 for overall determination of the phenolic content (F–C method), DPPH scavenging activity and ORAC tests in the extracts, and individual determination of naringin and hesperidin by a diode array detector (DAD) after LC separation (Khan *et al.*, 2010). A more recent study for USAE of the same raw material used LC–quadrupole time-of-flight (QTOF) for identification of 8 coumarins, 4 polymethoxyflavones, 2 flavonoidaglycons, 12 glucosylated flavonoids, and 7 further compounds not classifiable in the previous groups, thus providing useful information on the orange peel composition (Molina-Calle *et al.*, 2015).

In addition to USAE of antioxidant compounds from plants or foods, the USAE of water-soluble polysaccharides from dried and milled by-products generated from *Agaricus bisporus* production has recently been reported (Aguiló-Aguayo *et al.*, 2017), and also extraction of hemicelluloses from grape pomace (Minjares-Fuentes *et al.*, 2016), thus showing the wide field of little to unexplored applications of USAE in food processing.

It is worth emphasizing that authors working with LCs coupled to photometric detectors use most times HPLC analysis to designate both individual separation and molecular absorption detection.

2.2. Extraction of volatile compounds

It is well known that US application favors removal of volatiles from liquids as US cleaning baths have for years been used for this purpose (*e.g.*, for degasification of chromatographic mobile phases). Nevertheless, authors working on USAE of volatile components from saffron developed this step in an open atmosphere without protection for losses of both the target compounds, after transference to the liquid extractant, and the volatile medium (an ethanol–ethylacetate mixture). The authors stored the extract at 4 ºC in the absence of light until gas chromatography–mass spectrometry (GC–MS) analysis, by which they identified 40 saffron components (Jalali-Heravi *et al.*, 2009). More recently, a methanol–acetonitrile mixture has been used for USAE of volatiles from the same matrix, followed by dispersive liquid–liquid microextraction (DLLME) to isolate and enrich the extract on the target compounds prior to GC separation and MS detection (Sereshti *et al.*,

2014). No special operating conditions were used to avoid losses of the more volatile extract components, which could be the reason for the high detection and quantitation limits provided by the method (at the mg/L level) for the 27 identified compounds.

2.3. Multielement ultrasound-assisted extraction

Metal ions have been extracted by US assistance from food and beverages prior to determination by the proper atomic detector (Tadeo et *al.*, 2010). US-assisted cloud point extraction/preconcentration of metal ions from selected foods and beverages was achieved before determination of antimony and tin by flame atomic absorption spectrometry (FAAS) (Altunay *et al.*, 2016). A limit of detection for both Sb(III) and Sn(IV) of 0.03 mg/L and sensitivity enhancement factors of 114.5 and 110.6, respectively, show the usefulness of the method.

A multivariate optimization was applied to the development of a US-assisted multielemental extraction procedure for analysis of target inorganic ions in bean samples by inductively coupled plasma optical emission spectrometry (ICP-OES). The procedure consisted of treatment of the powdered samples with an acid mixture, then subjected to US energy to extract the target ions of Ba, Ca, Cu, Fe, K, Mg, Mn, Sr, and Zn prior to introduction of the extract into the instrument (Carvalho Santos *et al.*, 2009).

2.4. Extraction of undesirable compounds in food

A wide range of organic and inorganic contaminants is found in the environment as a consequence of diverse anthropogenic activities or natural processes. Their presence in the different environmental compartments may cause adverse effects on human health and animals, including cancer and/or disruption of the immune system. In addition, these compounds can persist in the environment and bioaccumulate along the food chain; therefore, their potential presence in foods requires to be investigated. The traditional sample preparation procedures previous to analysis have been widely substituted by those that are faster and more efficient based on US assistance. Thus, USAE methods for pesticides, polycyclic aromatic hydrocarbons, pharmaceuticals, heavy metals, among others, have been reported and reviewed (Tadeo *et al.*, 2010). An interesting example of USAE is that of 19 *Fusarium* toxins from muscle, liver, kidney, and fat of swine, bovine,

and sheep; muscle and liver of chicken; muscle and skin of fish, as well as milk and eggs. The extracts were then subjected to defatting with *n*-hexane and cleanup by solid-phase extraction (SPE) on BondElut Mycotoxin cartridges. All these steps provided an analytical sample to be injected into a reversed-phase LC coupled to an electrospray ionization triple quadrupole mass spectrometer for separation and detection/quantitation of the analytes. Despite the extraction step only required 15 min, the previous enzymatic hydrolysis step took 16 h. The latter could also be shortened by US assistance, as activation of hydrolytic enzymes has been widely demonstrated (Delgado-Povedano and Luque de Castro, 2015).

3. Special ultrasound-assisted extraction methods for food components

USAE methods with special characteristics, such as the use of ionic liquids (ILs) as extractants, formation of emulsions, development of derivatization reactions simultaneous with extraction or the use of biocatalyzed reactions, are the subject of this section.

3.1. Ultrasound-assisted extraction using ionic liquids

The boom of ILs proclaimed their advantages (namely, negligible vapor pressure, wide liquid range, good stability, tunable viscosity, good miscibility in water and organic solvents, good solubility and extractability for organic compounds, and suitability for energy dissipation (Du *et al.*, 2007)), which were later moderated and gave them an also moderated position in analytical applications. ILs were used as promising extractants of plant components such as alkaloids, terpene lactones, phenols, and quinones (Maet *et al.*, 2011). An example of ILs use is the USAE of four biphenyl cyclooctene lignans from the fruit of *Schisandra chinensis* Baill. Seventeen types of ILs with different cations and anions were investigated, and a 0.8 M 1-lauryl-3-methylimidazolium bromide water solution was selected as extractant. After optimization of US parameters such as US power, time for US treatment, and solid–liquid ratio by an RSM, the extracts were compared with those obtained by conventional extraction, resulting in an efficiency increase of about 350%, and a shortening of the process time from 6 h to 30 min. Both the nature of the cation and anion that constituted the IL showed to be influential on the extraction process (Ma *et al.*, 2011).

Task-specific Brönsted acidic imidazole ILs with two acidic sites have been designed primarily as an alternative to traditional mineral acids such as H_2SO_4 and HCl for catalytic applications (Wilkes, 2004), and as excellent extractants for preparation of natural products such as flavonoid glycosides (Yao *et al.*, 2012) or phenolic acids (Liu *et al.*, 2016). Recently, a method based on 3-methyl-1-(4-sulfonylbutyl) imidazolium hydrogen-sulfate as extractant and the synergistic effect of US and microwaves (MW) was used for the extraction of pectin from the albedo part of pomelo peels and the extracts compared with those obtained by conventional extraction with reference extractants. ANOVA application showed no significant differences in total carbohydrate content and degree of esterification (DE), while galacturonic acid content was significantly different for the pectin from each extractant. The differences revealed by images of atomic force microscopy (AFM) and SEM (Fig. 4) suggest changes of the physicochemical properties of pectin by the extractant that were not investigated using appropriate analytical instrumentation (Liu *et al.*, 2017), an aspect of paramount importance before the use of the extracted compounds as food components.

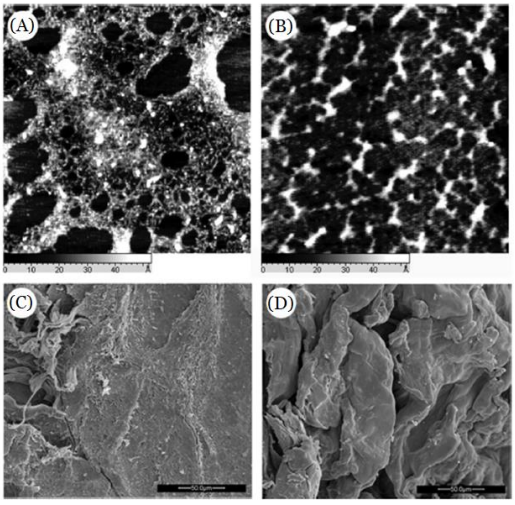

Fig. 4. AFM and SEM images of pectins. AFM images of pectin extracted with an aqueous solution of the selected IL **(A)**, and a hydrochloric acid solution **(B)**; SEM images of pectin extracted with an aqueous solution of the selected IL **(C)**; and a hydrochloric acid solution **(D)**. *AFM*, atomic force microscopy; *IL*, ionic liquid; *SEM*, scanning electron microscopy. With permission of Elsevier, Liu, Z., Qiao, L., Yang, F., Gu, H., Yang, L., 2017. Brönsted acidic ionic liquid based on ultrasound-microwave synergistic extraction of pectin from pomelo peles. Int. J. Biol. Macromol. 94, 309–318.

3.2. Ultrasound-assisted emulsification/extraction

The availability of US to form fine emulsions has been exploited in dealing with two main types of samples:

(1) With solid samples and using two immiscible polar/no polar extractants for simultaneous removal of both polar and no polar sample components. The approach known as US-assisted emulsification–extraction (USAEE) is based on the use of two immiscible extractants, which, under US application, form an emulsion that allows close contact of both with the solid sample and a rapid mass transfer of the compounds extractable to one or the other liquid phase with the help of the cavitation phenomenon, also promoted by US. Thus, USAEE is an effective extraction approach for subsequent characterization of the metabolic profile of a given solid sample, as proven for the extraction of polar and no polar compounds from samples such as acorns, grape seeds, or alperujo (Mason, 1991).

The excellent performance of USAEE with vegetable matrices led to the development of a method for extraction of as many components of orange peel as possible with a view on optimizing resources and shortening the extraction time. The method allows the simultaneous extraction of polar and no polar compounds by using an 80:20 (v/v) methanol–water mixture as polar extractant, and *n*-hexane as a no polar extractant. The US-assisted method facilitates both the formation of a stable emulsion between the two immiscible extractants and mass transfer from the solid sample to the liquid phases by a wide contact surface. Optimization of the USAEE by desirability conditions for both extracts led to analytical samples that were subjected to LC–QTOF MS/MS in positive and negative ionization modes and tentative identification of the most significant compounds present in each extract (a total of 51 compounds). Comparison of the USAEE extracts with those obtained by conventional extraction for polar and no polar compounds by principal component analysis showed that the polar extracts were similar in extraction efficiency, while the no polar ones showed clear differences. This behavior was attributed to the effect of US on lipids (Cañizares *et al.*, 2004).

(2) With liquid samples for liquid–liquid microextraction (LLME). In this case, a large volume of usually aqueous sample is put into contact with a very small volume of usually organic extractant that is finally emulsified with the sample under US application, thus giving place to UASE–LLME (Delgado-Povedano and Luque de Castro, 2013a). The

approach is useful for analytical purposes, but it is not suitable for automation, thus hindering its application in routine analysis, and in particular for larger-scale industrial applications (Delgado-Povedano and Luque de Castro, 2013a).

3.3. Ultrasound-assisted extraction and in situ derivatization

Ultrasound does not only affect heat and mass transfer, but can also induce chemical reactions, or at least enhance chemical reactions (*i.e.*, similar to the improvements in mass transfer, US can also have a synergistic effect on chemical reactions and as a consequence to the "removal" of the target extracted species by conversion into a product). Cavitation is the main US effect that favors both reaction and extraction (Delgado-Povedano and Luque de Castro, 2013b). Derivatizing reactions (*e.g.*, complex formation, ion-pair formation, depolymerization, redox, alkylation, esterification, addition, organics ethylation), oxidation (*e.g.*, involving inorganics, organics, organometallics or to study oxidative stability), or hydrolysis (*e.g.*, phenols, carbohydrates) are among the most used. Special care has to be taken to understand the influence of US on each step for an appropriate optimization of US variables. An example is found in the determination of phenols in virgin olive oil based on extraction of the target analytes into a basic aqueous medium containing the F–C reagent. In this case, only the hydrolysis reaction to form phenolate as previous step is favored by US, with no influence of this intervention on the derivatization reaction as such (Ruiz-Jiménez and Luque de Castro, 2003).

One example of the drastic reduction in time provided by a reaction assisted by US energy is a study reported on oil stability. The use of a probe and the appropriate optimization of the US variables have allowed Rancimat times of 129 h to be reduced to 50 min; therefore, the overall time required for the determination of oil stability, even for highly stable virgin olive oils, is shorter than 1 h (Cañizares *et al.*, 2004). The study also revealed that US is not a recommended type of energy to assist oil processes in which fat oxidation is not the target.

When developed in a continuous approach, USAE and in situ derivatization can be coupled to other steps of the analytical process such as online filtration, cleanup–preconcentration, individual separation, and detection (Delgado-Povedano and Luque de Castro, 2013b).

3.4. Ultrasound-assisted extraction and in situ enzymatic catalysis

The effect of US on the enzymatic activity is a debatable matter that requires in depth studies involving the US frequency and the biocatalyst structure, among the most significant. Examples of the controversial effect of US on biocatalyst behavior are found in the review by Delgado-Povedano and Luque de Castro (2015).

4. Comparison of ultrasound-assisted extraction methods with other methods using different energy assistance or dual energy assistance

The most common and immediate step after development of a USAE is its comparison with conventional counterparts. USAE has shown to exhibit advantageous characteristics. Furthermore, USAE methods have been compared with extraction methods assisted by other intervention approaches, or even with methods simultaneously assisted by two different types of energy.

4.1. Comparison of ultrasound-assisted extraction methods with conventional extraction methods

The extracts of anthocyanins from different fruits obtained by both a conventional stirring method and a USAE method were compared on the basis of total phenol content, total monomeric anthocyanin content, DPPH scavenging activity, and the analysis by LC–DAD using three commercial standards. The poor resolution of the analytical instrumentation hindered a critical comparison of the effect of the methods on the extracted components, and degradation was considered as "a decrease of extraction efficiency by increasing the time of US application" (Wang *et al.*, 2016).

Comparison of the efficiency in the extraction of total carbohydrates from *Stevia rebaudiana* Bertoni by conventional extraction and USAE showed an increase by a factor of 1.5, a lower processing temperature (68 *vs.* 100 ºC) and a drastic time reduction by using the latter method. The improvement in efficiency was explained by the mechanical action of the ultrasound on the cell walls that resulted in an increased accessibility and extractability of the target components; while a better quality of the extracts was attributed

to a better extractability of stevioside and rebaudioside A, the most desirable stevia components (Liu *et al.*, 2010).

The method proposed for USAE of carotenoids from pomegranate peel was compared with the conventional extraction method only in terms of efficiency and time required for extraction development, but the effects of US on the stability of the oils used as extractants were not taken into account (Kaderides *et al.*, 2015).

Comparison of Soxhlet extraction (SE) and USAE to remove vanillin from cured vanilla pods showed that polar extractants were the best in both cases and a temperature of 90–100 ºC in the former and ambient temperature in the latter provided almost similar efficiency for 8 and 1 h extraction, respectively. The effect of temperature and/or US on the target compounds or on the sample matrix were not studied (Jadhav *et al.*, 2009).

4.2. Simultaneous comparison of ultrasound-assisted extraction methods with several extraction methods

The extraction of 26 characteristic components covering four structure types (flavonoids, terpene lactones, ginkgolic acids, and phenylpropanols) in ginkgo seeds by US, refluxing, percolation, or SE were compared to determine the best extraction procedure for the target compounds. USAE proved to be better than the percolation and Soxhlet methods in terms of efficiency. Refluxing and ultrasonication were found to provide comparable results, but refluxing was a more time-consuming procedure (Zhou *et al.*, 2014).

4.3. Comparison of extraction by ultrasound combined with a different intervention technique with other extraction methods

The comparison of the extraction of oleuropein from olive leaves by USAE with reduced pressure, conventional extraction under atmospheric pressure, reduced-pressure boiling extraction, or USAE provided the highest efficiency and shortest extraction time for the first, which also produced the highest alteration of the leaf structure (Xie *et al.*, 2015). Nevertheless, when a method to extract bioactive compounds from papaya seeds based on high hydrostatic pressure extraction was compared with USAE and conventional extraction, the first also showed to be the most effective (Briones-Labarca *et al.*, 2015).

Superheated water extraction (SHWE) was assisted by US for removal of naphthoquinone pigments from purple gromwell, and the results thus obtained were compared with those provided by SFE with CO2 or by SE. The comparison showed that the extraction yield of naphthoquinone pigments by US–SHWE was the highest, up to 0.31%, while the yields achieved by SE and SFE were 0.21% and 0.19%, respectively; and the first also provided the extracts with less impurities and the best inhibitory effects on *Escherichia coli* and *Staphylococcus aureus*, followed by that with SE. However, the extracts obtained with SE showed the highest DPPH scavenging capacity and reducing activity (Yang *et al.*, 2013).

The combined effect of sequential application of US and MW (US–MAE) on the yield and DE of pectin from pomelo peel using citric acid was recently investigated (Liew *et al.*, 2016). Under the optimum working conditions (pH 1.80, 27.52 min ultrasonication followed by 6.40 min MW irradiation at 643.44 W), the yield and the DE of pectin obtained were 38.00% and 56.88%, respectively. Under the optimized US–MAE conditions, the pectin extracted by MW–USAE and by only USAE or MAE were studied and compared. The order of yield efficiency was US–MAE > MW–USAE > MAE > USAE. The pectin galacturonic acid content obtained by combined intervention techniques was always higher than that provided by a single technique, and the pectin gel from combined techniques exhibited a pseudoplastic behavior. The visual aspect in each case is shown in Fig. 5.

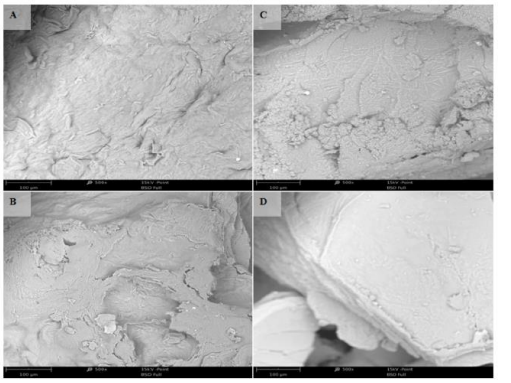

Fig. 5. SEM micrograph of pectin extracted by **(A)** USAE; **(B)** MAE; **(C)** MW–USAE; and **(D)** US–MAE at ×500 magnification, 100 mm. *MAE*, microwave-assisted extraction; *MW*, microwave; *SEM*, scanning electron microscopy; *US*, ultracound; *USAE*, ultrasound-assisted extraction. With permission of Elsevier, Liew, S.Q., Ngoh, G.C., Yusoff, R., Teoh, W.H., 2016. Sequential ultrasound-microwave assisted acid extraction (UMAE) of pectin from pomelo peles. Int. J. Biol. Macromol. 93, 426–435.

The influence of US on SFE to remove bioactive compounds from blackberry bagasse was also studied, and its effect (at 400 W, frequency not given) was significant at a pressure of 15 MPa, contributing to enhance the extraction kinetics and increase the global yield without compromising the quality of the extracts. The enhancement of mass transfer by US is caused by changes in the structure of the substrate, as can be observed by the image analyses in Fig. 2. Specifically, the increase of particles stuck onto the surface of blackberry bagasse contributed to reduce the barriers to both supercritical fluid and extractable compounds (Pasquel Reátegui *et al.*, 2014). When compared with SE with ethanol as extractant, this provided higher yield, which is attributed to the higher polarity of ethanol as compared with CO2, even in the presence of water or ethanol as cosolvent.

5. Scaling up laboratory methods for ultrasound-assisted extraction of food components

The use of US in the food industry is not exclusive to extraction. In fact, there are a number of food processes that profit from this technology to shorten or improve certain applications and, in some cases, even facilitate phenomena that cannot be achieved by any other means. Some examples include but are not limited to filtration, defoaming, depolymerization, degassing/deaeration, cooking, cutting, demolding and extrusion, freezing and crystallization, drying, defrosting/thawing, brining, pickling and marinating, meat tenderization, separation (emulsion breaking), emulsification/homogenization.

When considering US for extraction purposes, it is essential the laboratory development has to be exhaustive, particularly in identification of the extract components and in the potential degradation caused or favored by US energy. Only then upscaling to pilot scale and eventually industrial scale should be considered. The absence of degradation must also be assessed at the pilot plant and industrial scales.

Residues or waste valorization has been the most prominent application of the USAE method over the last decade. Extracts rich in antioxidants, mainly phenol compounds (usually named as polyphenols, despite some of them are simple phenols as is the case with hydroxytyrosol, tyrosol, oleocanthal, etc.), have been obtained from apple pomace (Virot *et al.*, 2010; Pingret *et al.*, 2012), from chicory grounds (Pradal *et al.*, 2016), olive leaves or olive oil pomace (Delgado-Povedano *et al.*, 2017), and pomegranate peels

(Kaderides *et al.*, 2015). Usually the residues are richer in antioxidants than the main product (cider or virgin olive oil, for example).

Experiments at pilot plant scale have ratified the results obtained at laboratory scale (shortening of the extraction time, enhancement of the final yield, low operating temperature) (Cárcel *et al.*, 2012). The final aim at the industrial scale of reaching the working conditions to obtain a given yield with minimal process duration and/or minimal energy consumption was in most cases achieved.

Fig. 6. Industrial ultrasonic equipment (capacity: 50, 500, and 1000 L). With permission of Elsevier, Chemat, F., Huma, Z., Khan, M.K., 2011. Applications of ultrasound in food technology: processing, preservation and extraction. Ultrason. Sonochem. 18, 813–835.

Despite the food industry being reluctant to apply new extraction technologies, such as USAE, a better understanding of US energy and assessment of the quality of the extracts thus obtained are displacing the old traditional systems in the industry for US-assisted equipment, as shown in Fig. 6.

6. Future trends in ultrasound-assisted extraction of food components

A desirable step in the development of an extraction method based on US assistance (as should be with any other type of technology intervention) is exhaustive identification of the extract components to detect any potential degradation. The nature of the degradation products is of paramount importance when the extracts are used to supplement foods or as nutraceuticals. Once the healthy nature of the extracts has been assessed, the US conditions (*i.e.*, US frequency, power, and application time) to obtain the extracts must be maintained when the process is upscaled.

A variable rarely taken into account by US users is frequency, the importance of which is often ignored in publications. To study different frequencies of US devices can be very useful to avoid degradation and/or obtain the pursued results.

"Bioactive" is a catch word applied to a number of plant components that are presently extracted with US assistance (Kadam *et al.*, 2015). Additional research is required, including upscale studies to facilitate the adoption of US in the bioactive compounds industries to enhance processing efficiency and capability after in-depth study of extractants, thermolability, and fragility against US action, particularly against free radicals formed in polar media. Usually, intermittent US application (short duty cycles) does not affect the molecular structures of the target compounds.

While multivariate optimization designs are useful to reduce the number of experiments, univariate optimization of some parameters is of paramount importance to assess the effect changing process variables has on the target components (Sourki *et al.*, 2017). Multicriteria optimization and desirability criteria are of interest in USAE and should be promoted (Tomšik *et al.*, 2016).

A more in-depth knowledge of USAE at the laboratory scale is the necessary step to potentiate the interest in upscaling these methods, taking advantage of their positive aspects at industrial scale.

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Chapter 11

Ultrasound: A subexploited tool for sample preparation in metabolomics

Ultrasound: A subexploited tool for sample preparation in metabolomics

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Ultrasound: A subexploited tool for sample preparation in metabolomics

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Abstract

Metabolomics, one of the most recently emerged "omics", has taken advantage of ultrasound (US) to improve sample preparation (SP) steps. The metabolomics–US assisted SP step binomial has experienced a dissimilar development that has depended on the area (plant or animal) and the SP step. Thus, plant metabolomics and US assisted leaching has received the greater attention (encompassing subdisciplines such as metallomics, xenometabolomics and, mainly, lipidomics), but also liquid–liquid extraction and (bio)chemical reactions in metabolomics have taken advantage of US energy. Also clinical and animal samples have benefited from US assisted SP in metabolomics studies but in a lesser extension. The main effects of US have been shortening of the time required for the given step, and/or increase of its efficiency or availability for automation; nevertheless, attention paid to potential degradation caused by US has been scant or nil. Achievements and weak points of the metabolomics–US assisted SP step binomial are discussed and possible solutions to the present shortcomings are exposed.

Keywords: ultrasound, sample preparation, metabolomics, metallomics, lipidomics, xenometabolomics.

1. Introduction

Analytical methods typically consist of a number of steps (from sampling, to sample preparation, isolation of the target compounds if required, identification, quantitation and, finally data handling) each of them with its specific weight on the final results. A glance to the nature and goal of most sample preparation operations allows evaluation of the key influence of these steps on both the total time required to complete the analysis and the quality of the obtained results. However, it has only been in recent years when this step has risen to the prominent place that it holds at present within the analytical process [1,2].

The disciplines appeared in recent years under the umbrella of the common subfix "omics" started in 1986 with genomics [3], and reached its present completness in 2002 with metabolomics, defined that year by O. Fiehn as "a comprehensive analysis in which all the metabolites of a biological system are identified and quantified" [4]. The general concept of metabolomics as "the omics of small molecules" leads to the subsequent definition as "the comprehensive analytical approach to study all low-molecular weight species (typically defined as <1000 or <1500 Da) present in a given biological system of interest". Recently, Ryan *et al.* [5] seem to lean toward a definition that encompasses characterization and quantitation of all metabolites within a sample, but with the qualification that metabolomics considers both spatial resolution (due to cellular compartmentalization of metabolism) and temporal changes in response to environmental stimuli (the latter should be extended to xeno stimuli).

The joint use of the information provided by two or more omics constitutes the systems biology approach [6].

Metabolomics analyses encompass different strategies, which depend on the information required from the system under study [7], namely: (a) target analysis [8], which aims at qualitative and quantitative study of one or, more frequently, a small group of chemically similar metabolites; (b) global metabolomics profiling [9], which allows detection of a broad range of metabolites by using a single analytical platform or a combination of complementary analytical platforms —based on GC–MS, LC–MS, capillary electrophoresis (CE)–MS or nuclear magnetic resonance (NMR)— to obtain a comprehensive profile of the metabolome; (c) metabolomics fingerprinting [10], a high throughput, fast methodology for analysis of biological samples that provides fingerprints for sample classification and screening; and, (d) metabolomics footprinting [11], which

aims at the study of metabolites in extracellular fluids, also known as exometabolome or secretome. Obviously, the complexity of sample preparation for each strategy is different increasing from (d) to (a). The most recent trend leans to simplification by establishing distinction only between targeted and untargeted analysis including profiling, which require quite different sampling and sample preparation approaches [12].

The physical state of the samples in metabolomics studies encompasses from solids —mainly in plant metabolomics, and sporadically in animal and clinical metabolomics— to gases —exhaled breath or aromas. Therefore, the operations to be developed prior to introduction of the analytical sample [13] either into high resolution separation equipment or into the detector are very varied and most of them can be accelerated/improved by auxiliary energies.

Ultrasound (US) is an auxiliary energy in analytical chemistry the effects of which have been differently used in general [14] and poorly in omics in particular. Despite the traditional use of US probes to disrupt cells in proteomics studies [15], and its recent application to study thermal aggregation, gelation, foaming and emulsifying properties of egg white proteins [16], US does not participate of the massive use of microwaves (MW) in proteomics [17]. The dissimilar use of these energies in analytical applications in general is a consequence of several factors:

(i) The use of cleaning US baths as analytical devices for which they have not been designed.

(ii) The comparison of the performance of cleaning US baths with that of specific designed MW-assisted analytical devices (particularly for digestion or leaching), in which the supremacy of the latter was most times demonstrated.

(iii) The nil importance given by the users to US frequency. Most US devices (including cleaning baths, probes and reactors) work within a small range of frequencies (*viz.* 20–40 kHz) despite power frequencies encompass the range 20–100 kHz. Each device usually works at a fixed frequency. After application of US by a given device, the users conclude US produces a nil, small or medium effect; sometimes without specifying the frequency of the US device they used [18].

The unfear opinion on the role of US energy to help analytical steps in metabolomics calls for: (i) examples of typical metabolomics publications in which US has been used to favor one or several steps; (ii) critical discussion of US aspects that wether

have not been taken into account or have been misinterpreted by the authors; (iii) ways to improve and extend US applications in metabolomics, thus taken profit of this energy.

2. Generalities on the use of US in metabolomics

In the light of the research so far developed in the field of metabolomics, it is clear that US has been mainly used in target analysis (*e.g.*, determination of estrogens in human urine [19], simultaneous determination of quinocetone and three of its synthesized desoxy metabolites in swine urine [20], or carbamate pesticides in environmental water samples [21]). Presently, this energy is starting to be used in other strategies, as global metabolomics profiling (*e.g.*, metabolomic analysis of clinically relevant bacteria —*Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli, Yersinia pseudotuberculosis* or *Methicillin-Sensitive Staphylococcus aureus*) [22], and metabolomic profiling of human saliva [23]), which highlight US subexploited utility.

The fact that US application does not increase the temperature of the system is one of the reasons why this energy is a suitable option to work with thermally unstable metabolites present in a biological fluid, cell or organism under a given set of physiological conditions. Benzodiazepines in human plasma [24], vitamin C in dehydrated carrots [25] or β-carotene in *Spirulina platensis* [26] are examples of this behavior. US is also a suitable option when working with polar and no polar metabolites/samples, because US, in contrast to MW, does not require polarity of sample components to promote interaction with them. Nevertheless, US is not the panacea in dealing with auxiliary energies, as the formation of free radicals by the cavitation phenomenon in polar media can constitute a shortcoming for some given systems.

As commented before, the most frequently used US devices are ultrasonic cleaning baths and probes (the use of ultrasonic reactors shyly starts at present). The former (designed for cleaning glassware and degassing) are the most widely used in metabolomics sample preparation, because they are the most affordable and cheapest ultrasonic laboratory devices available in the market. This is why US cleaning baths are used for analytical tasks which require more specifically designed US equipment. The main drawbacks of US cleaning baths, which are the sources of their irreproducibility, are the decline of power with time and the lack of uniformity in the transmission of US. Despite these unfavorable aspects, these devices have sometimes provided the best results for

leaching as compared with both Soxhlet and maceration methods, probably due to higher temperatures of the Soxhlet method and longer time required for maceration (up to 18 h) [27]. Nevertheless, a probe ultrasonic device, either immersed in the sample or in a water bath where the flask containing the sample is located, is the most desirable option [28].

In dealing with the approaches designed for US assisted sample preparation in metabolomics they have been either of the continuous or discontinuous type. The former approaches are endowed with the capacity for being coupled to equipment where subsequent steps of the analytical process are developed (sometimes, a US device has assisted several analytical steps by accelerating/improving them [29]). These approaches that undoubtedly increase automation of sample preparation have preceded liquid or gas chromatographs and even CE equipment [30]. The design of discontinuous approaches is simpler and allows applications difficult or impossible to carry out by those of the continuous type, as is the case with steps based on emulsification–extraction, either involving solid–liquid or liquid–liquid systems [31].

Two main aspects must be taken into account in dealing with the use of US in metabolomics so far:

(i) Most authors working on target metabolomics both in plant and animal or clinical metabolomics do not consider their research within this omics; therefore, they use the terms antioxidants, phenols, bioactive compounds, lipids (individual or overall analysis), hormones, etc., but the word metabolite(s) does not appear in the publication.

(ii) The general use of high power US is not accompanied by assessing no degradation effect of this type of energy, despite the claim “no degradation effect was detected” that in general refers to as “no degradation effect was visually observed”.

The number of different steps in metabolomics sample preparation assisted by US forces to discuss them in the order they are included in the analytical process, starting with preliminary steps, solid samples and the different actions of US in them, and liquid samples, from liquid–liquid extraction (LLE) to derivatization without forgetting enzymatic reactions.

3. Preliminary US assisted steps in metabolomics

The use of US to improve steps in metabolomics that precede the socalled sample preparation [14] has been very varied.

US has been used to improve freezing and crystallization processes prior to evaluation of the viability of *Lactobacillus plantarum* subsp. *plantarum* by Kiani *et al.* [32]. This energy induced nucleation in the supercooled suspension of L. *plantarum*, thus maintaining highly viable bacteria cells in frozen products and reducing damage caused by freezing. Ultrasonication during nucleation or phase change of the freezing process holds promising as a tool to ensure viability of frozen suspended cells.

Freezing–drying processes can also take advantage from US application that allowed supplying additional sublimation energy without sample heating in the case of red bell pepper [33]. Application of US led to temperature increase, which was reduced when this energy was applied as pulses (in this case, 10 s US application and 90 s recovery phase), which made possible freeze-drying at increased sublimation rates without causing sample heating during the 7 h required by the process. Application of US did not affect product quality of the lyophilized sample in terms of ascorbic acid content, bulk density, color, and rehydration characteristics.

Also, the influence of US on the microstructure of tomate pulp, lycopene content and *in vitro* bioaccessibility has been studied [34]. US was responsible for loss of tomato cell integrity, as well as partial de-esterification of tomato pectins. In contrast, ultrasonicated tomato pulp had greater gel like properties than a no ultrasonicated sample. The authors deduced that the partially de-esterified pectin molecules gave rise to a new molecular organization leading to the formation of a stronger network with increased gel like properties. Such an effect on the tomato pulp structure resulted in a decrease in lycopene *in vitro* bioaccessibility of the ultrasonically treated tomato pulp. In this study, in which the authors always worked at a fixed frequency of 24 kHz and a temperature never exceeding 90 °C, undesirable effects such as loss of tomato cell integrity and decrease in the degree of pectin esterification could be avoided by using lower temperatures and, especially higher frequencies —with the concomitant effect of reduced cavitation.

As commented before, US is not always the panacea, as this type of energy also caused undesirable effects, mainly owing to degradation. An example is the use of this energy to modify cell structure and mass transfer in potato tissue [35]. Cell disruption

caused by US (24 kHz) was limited to a thin tissue layer in direct vicinity to the sonicated surface, but mass transfer was influenced throughout the complete sample (a cylinder of 12 mm diameter and 1 cm height). Hence, US affects mass transfer at overall tissue level. The authors used conductivity to measure cell injury as the amount of ions leaking from a treated tissue in relation to the total amount of present ions. Modification of sample metabolites by ultrasonication was not a matter of study in this research. On the contrary, Zuo *et al.* studied the damage on potato starch granules induced by US [36]. The treatment of granule suspensions by power US caused damage to the starch surface and, under certain working conditions, shattering of the granules. The extent of damage to starch granules dispersed in water was first only slightly dependent on sonication power, then increased markedly by increasing this variable up to a given power threshold. Frequencies higher than 20 kHz were not assayed; therefore, the unknown behavior of the sample under higher frequencies should be studied, as well as under different temperatures as the authors worked at constant temperature of 5 °C.

On the other hand, Gamboa-Santos *et al.* have treated to use the degrading effect of US to favor desirable processes such as carrots blanching [25]. After assisting this process by US, the authors evaluated the content of vitamin C and sensory properties of the treated carrots. Fresh or dried carrots subjected to ultrasonication by a US probe (20 kHz, 400 W directly applied to the sample for 10 or 15 min at temperatures of 60 or 70 °C, respectively) showed vitamin C retention values lower than 4%; whereas carrots blanched conventionally using water at 95 °C for 5 min, boiling water for 1 min or in an autoclave for 2 min showed percents of vitamin C retention between 20.6 and 85.0. The main aim of this research was the use of a Headspace ChemSensor System (considered as an MS electronic nose) to obtain mass spectral fingerprints for sample classification. More in depth studies using different US frequencies would be of interest to know if negative effects such as vitamin C degradation can be avoided at higher values of this variable.

Particular individual uses of US in metabolomics or proteomics leads to consider its potential joint application in systems biology to facilitate development of preliminary steps. Some of these individual uses in proteomics are commented below.

US energy has demonstrated to enhance cholesterol removal ability of lactobacilli. US application (30 kHz, intensities of 20, 60 and 100 W and during 1, 2 and 3 min) influenced the properties of cellular membrane, increasing cellular viability immediately after treatment, except after treatment at 100 W and the longest application time (3 min),

owing to disruption of membrane lipid bilayer, cell lysis and membrane lipid peroxidation occurring under these conditions [37].

On the other hand, US has been used to treat whey protein coatings on frozen Atlantic salmon. Ultrasonication was applied at a fixed frequency of 35 kHz and three sonication times (1, 15 and 60 min). The drip loss after chilled storage, drip loss in thawing, cooking loss, thaw yield and yield were not affected by US as compared with salmon samples with untreated protein coatings. However, the authors observed decreases of the above parameters in samples from whey proteins sonicated 60 min in relation to those sonicated for 15 min. US application to the coatings delayed the lipid oxidation of salmon pieces in comparison with those coated with untreated proteins. In addition, US application increased whiteness of cooked samples and lightness, but did not modify the color of frozen and thawed fillets in comparison with untreated protein coated samples [38].

4. US assisted digestion in metabolomics

Digestion is not a common treatment in metabolomics as most sample preparation steps in dealing with solid samples use leaching as the way to obtain a treated sample containing the target metabolites and with lower undesirable matrix components than those digested. In fact, digestion of any type of tissue has been mainly used to liberate metals; therefore, metabolomics subdisciplines such as ionomics or metallomics should be those taking benefits from US assisted digestion.

An example of the positive effects of US for digestion as sample preparation in these metabolomics subdisciplines is the method reported by Cespón-Romero and Yebra-Biurrun [39], who used US (applied by an ultrasonic bath for 3 min at room temperature) to accelerate urine sample digestion for subsequent determination of trace metals such as copper, iron, manganese and nickel. Urine volumes of few mL were mixed with HNO_3 and digested under US application by immersion of the digestion unit into the US bath. It is remarkable that the authors do not specify the frequency and other characteristics of the US device they used.

5. US assisted leaching in metabolomics

Leaching or solid–liquid extraction is the sample preparation step in metabolomics

that has been more frequently improved, either enhanced and/or accelerated, by US energy. Batch and continuous approaches in meso and micro format have been used with these aims. Distinction is made between the plant and animal field, and the metabolomics subdiscipline to which the research belongs, if appropriate.

5.1. US assisted plant leaching in metabolomics

This is the area in which US has been more frequently applied to favor leaching of a wide number of metabolites, despite the word metabolite(s) does not appear in most of the publications on the subject, as commented previously. Lipids and other bioactive compounds such as antioxidants have received the highest attention. Examples of leaching prior to the determination of metals in living organisms are first discussed as they required the simplest analytical approaches.

5.1.1. US assisted plant leaching in metallomics

Metals in the plant kingdom can exist as constituents of the given plant (as is the case with copper in tomatoes); therefore, their study belongs to ionomics or metallomics, depending of the oxidation state in the target plant. Also the presence of the metal in the sample can be the result of exposition to contamination: in this case the study pertains to xenometabolomics. Examples in which US has been used in metallomics to facilitate leaching are as follows:

A dynamic, automated US-assisted system as that shown in Fig. 1A was used to assist leaching of essential macronutrients (calcium and magnesium) and micronutrients (copper, iron and zinc) from animal feeds. The overall system consisted of: (i) a leaching chamber where the sample was located and through which a leaching carrier (10% aqueous HNO_3) was circulated and the direction of which changed each 80 s, thus minimizing both dilution of the leachate and increased compactness of the sample in the chamber that could cause overpressure in the system; (ii) a programmable peristaltic pump; (iii) coils of enough length to keep the leaching carrier/leachate within the dynamic system during the go–back sequence; (iv) a US probe (duty cycle 0.8 s/s, output amplitude 80% of the nominal amplitude of the converter applied 20 kHz and power 400 W with the probe placed 1 mm from the top surface of the extraction cell); (v) a water bath at 40 °C in which the chamber

and the US probe were immersed for proper transmission of US. After leaching, the target metals were quantified by flame atomic absorption spectrometry (FAAS) [40].

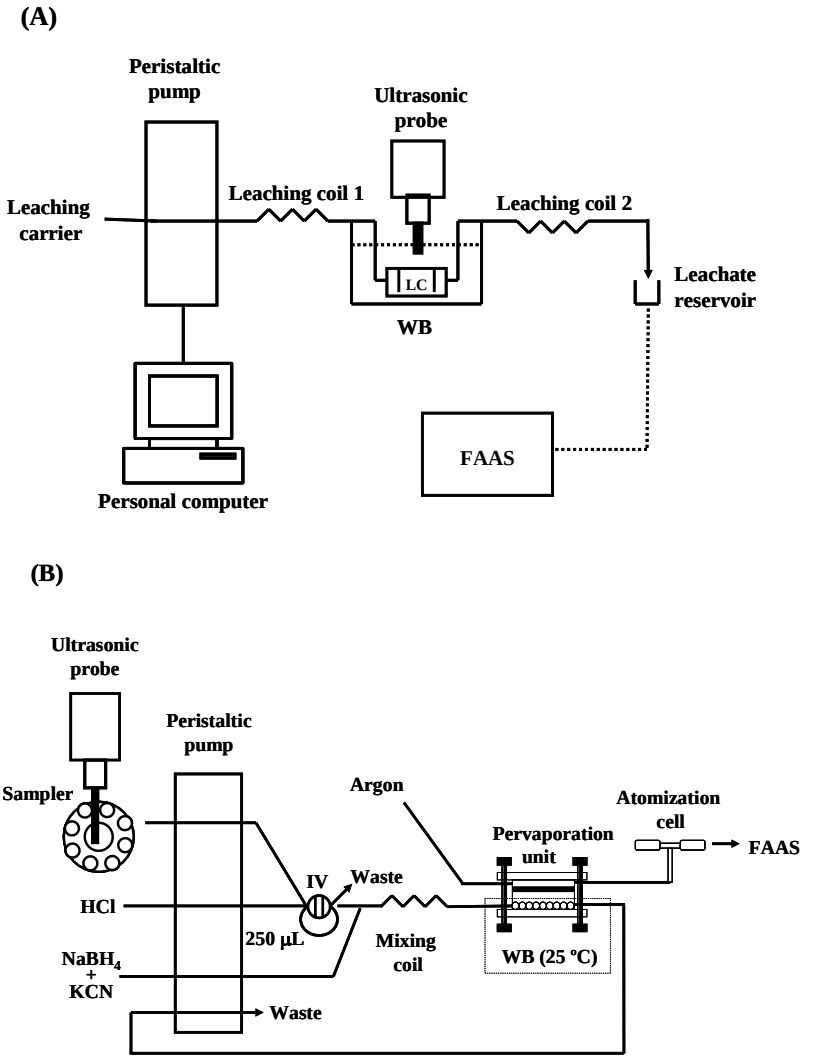

Fig. 1. Experimental setups for dynamic USAL and determination of metals. **(A)** For macro and micro nutrients in animal feeds. **(B)** For cadmium in vegetables by slurry formation USAL, hydride generation, pervaporation and atomic absorption detection. FAAS, flame atomic absorption spectrometer; IV, injection valve; LC, leaching chamber; WB, thermostatic water bath. (With permission of Springer-Verlag and Elsevier, Refs. [40] and [41], respectively).

A more complex dynamic approach was used for determination of cadmium in olive leaves (Fig. 1B), because after leaching the target analyte by an 1 M HCl + 16% (m/v) H_2O_2 aqueous solution with the help of a US probe acting for 17 min (duty cycle 0.9 s/s and output amplitude 100%) at the maximum power of the US probe, generation of the corresponding hydride and pervaporation of the volatile product was carried out prior to determination by FAAS [41].

5.1.2. US assisted plant leaching in lipidomics

US has been widely used to improve leaching of a wide number of metabolites as triglycerides and fatty acids thus constituting a key sample preparation step in lipidomics. Although most samples subjected to US assisted leaching (USAL) have been seeds, also flowers and plants have been the target of this step. In this context, USAL has been compared with other conventional techniques as Soxhlet extraction (SE) or supercritical fluid extraction (SFE) and in all instances USAL was found to shorten the leaching time for the same or similar target metabolites.

A wide variety of lipids has taken advantage of USAL, as is the case of phospholipids (PL) from palm-pressed fiber. The leaching efficiency of PL increased by increasing the sonication time and decreased when the amplitude and cycle increased. Maximum PL leaching efficiency was achieved by 30 min sonication, 20% US amplitude and 0.2 (W/s) cycle with an 1:4 (g/mL) solid–liquid ratio using a US device working at 24 kHz. The authors deduced by SEM images that US caused disaggregation and rupture of the plant cell walls, thereby facilitating leaching of PL from the target fiber [42].

USAL for plant sample preparation in lipidomics has been compared with either traditional and no traditional leaching. An example of the former case is the extraction of grape seed oil, which required 30 min of USAL at 20 kHz, 150 W to obtain a 14% (w/w) yield, while a similar result by SE required 6 h. No significant differences were observed for the major fatty acids obtained by both methods [43].

When the leachates of fatty acids from quince seeds obtained by USAL were compared to those provided by SFE a similar lipid composition was obtained [44]. An exhaustive optimization of inter-related variables in both methods (*viz.*, 30 min sonication time, 12 mL of *n*-hexane, 55 °C in USAE; and 353 bar of CO_2 pressure, static extraction time for 10 min, dynamic extraction time for 60 min, 150 µL of methanol as modifier, at 35 °C

in SFE) allowed obtaining a maximum oil recovery of 19.5% by USAL, and 24.32% by SFE, mainly constituted in both cases by linoleic, palmitic, oleic, stearic and eicosanoic acids. The extracts from both methods were subject to transesterification and GC–MS analysis, which showed only slight or negligible differences in methyl esters profiles of the extracted oils. It is remarkable that better efficiency is obtained by SFE at the expenses of a longer, complicate and expensive process. In neither case the authors studied potential lipid degradation.

On the contrary, the quality of oil from Jatropha curcas and Pongamia seeds (both of Indian origin) by different methods (USAL, SE and direct reflux) was studied by Kaul *et al.* who analyzed in all cases the triglycerides and fatty acids by HPLC–DAD and finally supported the results on the determination of the acid value of the obtained oil by the ASTM D-974/04 method [45]. Unfortunately, molecular absorption was not the most appropriate technique to identify degradation caused by the different working conditions of each method. Significant differences concerning the extraction time and acid value of the oil (mg KOH/g) were obtained, as US application shortened the time required for extraction from 16 h (by conventional methods) to 3 min (by USAL), and allowed obtaining oils with acid value lower than by conventional methods.

A number of methods have recently been developed to extract aromas or essential oils from different plants. An example was provided by Goula [46], who carried out USAL to obtain oil from pomegranate seeds, using *n*-hexane as leachant. The leaching yield of oil increased with the increase of leachant–solid ratio and US amplitude and decreased seed particle size, extraction temperature, and pulse duration/pulse interval ratio. Maximum extraction yield (about 60%) was achieved by a 20:1 extractant–solid ratio, 60% US amplitude, 0.2 mm particle size, extraction temperature of 20 °C and pulse duration/pulse interval ratio 5/15 using a US device working at 20 kHz. The yield thus achieved was higher than those reported by methods based on SE, SFE, normal stirring, microwave-assisted extraction (MAE) or cold pressing [47–49]. The authors explained the improved oil yields in terms of cavitational effects caused by application of high power US (130 W).

Also USAL can precede hydrodistillation of essential oils as a way to accelerate and improve removal from the solid; then taking profit from the volatility of these compounds to obtain a purer composition by leaving in the leachate no volatile compounds. This was the case with USAL of caraway seeds by using 1.5 L of water and an ultrasonic reactor acting for 30 min at 25 kHz and 1 W/cm. Subsequent hydrodistillation yielded 1.72% of the target

oils, higher than no sonicated samples (with yield 1.68%) and in a shorter time (30 min *vs.* 90 min). Identification of 21 components in the hydrodistillate by GC–MS showed no differences in composition between sonicated and no sonicated samples [50].

More appropriate for aromatic oils because of their volatility is the use of a closed, dynamic and automated US assisted system, as that shown in Fig. 1A for macro and micronutrients leaching [40]. The leachates obtained from laurel, rosemary, thyme, oregano and tuberose under the optimum working conditions in each case were subject to LLE coupled to either GC–flame ionization detection (GC–FID) or GC–MS for detection and identification of the extracted compounds, respectively. Only 10 min were required to obtain leaching efficiencies that greatly surpassed those provided by steam distillation or superheated liquid extraction (SHLE) for these oils, with higher quality of the extract. The leaching time of the proposed method was between 165 and 176 min (depending on the target raw material), shorter than steam distillation (more than 4 h) and between 31 and 20 min shorter than SHLE [51].

A promising, innovative study reported by Araujo *et al.* [52] compared five leaching methods (*viz.*, Chen, Folch, Hara and Radin, and Soxhlet methods, and the Bligh and Dyer method both conventional and US assisted) for lipids from *Chlorella vulgaris* to take advantage of the ability of this microalgae to grow rapidly, synthesize and accumulate large amounts of lipids (approximately 20–50% of dry weight). The authors also studied the addition of silica powder to evaluate the introduction of more shear stress to the system as to increase the disruption of cell walls, but the addition did not improve oil extraction. The Bligh and Dyer method assisted by US (40 kHz and 2.68 W/m) provided the highest extraction of oil from *C. vulgaris* (52.5% w/w), thus demonstrating the usefulness of this type of energy also in USAL from this matrix.

5.1.3. US assisted leaching of plant no lipid metabolites

The number of applications of US to favor leaching of metabolites different from lipids in dealing with plants drastically surpass other types of matrices, and, among metabolites, phenols and antioxidants in general have been the most frequent targets. The final use of the leachates establishes both the optimum features of the method and the analyses to be carried out. When the final aim of the research is to study global characteristics of the leachates such as total phenolic content (TPC), antioxidant capacity

or scavenging activity the toxicity of the leachant is not a limitation. One of the most used global methods for TPC is based on the Folin–Ciocalteu reagent, which has been applied to USAL leachates from different plants as apple pomace [53], arecanut [54], black chokeberry [55], olive leaves [56] or grapes [57]. In the last case, other general methods such as that for total amount of anthocyanins and total amount of tannic components have also been applied to the leachate.

Total antioxidant activity (AOA) has most times been determined by the ferrous reducing antioxidant power assay (FRAP), as is the case of leachates from arecanut [54] or marjoram [58]; while a common method to determine the scavenging activity is based on 2,2-diphenyl-1-picrylhydrazyl, applied to leachates from black chokeberry fruits [55].

In all overall studies, higher values of the given parameter were obtained when the leaching step was assisted by US as compared with traditional leaching methods. The authors attributed this behavior to a higher efficiency of the USAL step without checking possible degradation in the presence of power US that could yield products with higher values of the parameter under study. Research on this subject should involve, (i) appropriate analytical instrumentation to identify the compounds in the leachate, thus detecting potential degradation; (ii) use of higher US frequencies to avoid degradation if undesirable.

A recent example of incipient, but not sufficient, use of present analytical equipment is the research by Ahmad-Qasem *et al.* on phenol composition of olive leaves [56]. The authors used a dynamic recirculating system for a strict control of the temperature and a calorimetric procedure to determine the effective ultrasonic power transferred into the medium for every condition tested, but they do not specify the frequency of the US device used. First, the kinetics of the leaching step was monitored in the leachates by global parameters such as AOA by the FRAP method, Trolox equivalent antioxidant capacity (TEAC), and the TPC by the Folin–Ciocalteu method; then, they separated and identified the main phenols in the leachates by comparing the retention times, UV spectra and MS/MS data obtained by LC–DAD/MS–MS with those from either authentic standards or data reported in the literature. Remarkable in this research is the fact that the leaching time was shortened from 24 h (conventional method) to 15 min without modification of the leachate characteristics.

When the aim of the research was targeted metabolomics, a single compound or groups of compounds with common or different characteristics are quantified using

specific methods with or without prior identification depending on available both analytical equipment and standards. Such is the case with five isoflavones leachated from *Iris tectorum* Maxim [59], four acetophenones from *Cynanchum bungei* Decne [60], or eight ginsenosides from ginseng root [61]. Drastic reduction of the leaching time was highlighted in all instances, thus opening a door to potential implementation at an industrial scale.

When a single leached compound is the aim of the research, a more in depth study of the steps involved in the overall method has been carried out. Such is the case with hesperidin from *Citrus reticulata* Penggan peel [62]. Several US frequencies (20, 60 and 100 kHz) and four US powers (3.2, 8, 30, 56 W) were used to study potential degradation effects, which also were checked by subjecting a hesperidin standard to the leaching working conditions. Hesperidin yield under 60 kHz was the highest, but all three assayed frequencies provided yields that clearly surpassed that obtained by SE. No degradation of the target metabolite occurred by increasing the sonication time and methanol was the leachant providing the best efficiency; nevertheless, its use should be seriously considered before a potential application of the leachates for human or animal consumption.

Also bioactive glycosides (*i.e.*, crocins and picrocrocin) from *Crocus sativus* L. dry stigmas [63] have been leached using methanol as extractant and an ultrasonic probe under optimum conditions with a halved leaching time as compared to magnetic stirring according to ISO 3632-2. Degradation of crocins observed within the optimum time was rather low, the authors claimed, despite they used no appropriate analytical tools, but LC–DAD, to support this assertion.

Ionic liquids as leachants for USAL have been scarcely reported, but they have looked very promising for removal of three terpenoids indole alkaloids from *Catharanthus roseus* [64], using (1-allyl-3-methylimidazolium)Br. A soaking step prior to USAL increased diffusion of the leachant into the cellular structure, thus allowing improved solubilization of the metabolites. Compared with ME, USAL provided higher extraction efficiency (94.84±4.45% *vs.* 45.40±2.10%) and a significant shortening of leaching time (30 *vs.* 24 h).

Simultaneous USAL of polar (phenols) and no polar (lipids) from plants was achieved by formation of an emulsion with two immiscible leachants (methanol–water as dispersed phase and *n*-hexane as continuous phase). US enhanced formation of methanol–water microdroplets 1–15 μm in size, which act as solid–liquid microextractors spanning a

large surface area. The efficiency of this dual sample preparation approach was assessed by characterization of natural products of variable hardness including acorns, grape seeds, and alperujo, which provided quantitative leaching from acorns and alperujo in 9 min and in 20 min from grape seeds. Following leaching and separation of the two phases by centrifugation, the polar and no polar fractions were analyzed by HPLC–DAD and GC–MS, respectively. The method provided extraction efficiency similar to the Folch method (reference mild method for fat extraction, 4.5 h) in a shorter time, and extraction efficiency equal to or higher than the stirring-based method (reference method for phenol compounds extraction, 24 h) [65].

5.1.4. US assisted plant leaching in xenometabolomics

Xenometabolomics is the metabolomics subdiscipline that deals with the study of no endogenous compounds (*e.g.*, drugs and its metabolites, contaminants, dietary components, medicinal herbs, cosmetics) in a tissue or biofluid. Sample preparation in this omics has also taken profit from US, particularly in the plant field. Methods for haloacetic acids (HAAs) and metabolic pathways of either herbicides or others affected by them are examples of this assertion.

HAAs, included into the category of "disinfection byproducts", are mainly in water, but they also contaminate vegetables by irrigation. A continuous US assisted approach to favor extraction of nine HAAs from vegetables with *in situ* derivatization to methyl esters for subsequent analysis by GC–electron capture detection was developed by Álvarez-Sánchez *et al.* [66]. Optimization of simultaneous leaching and derivatization enabled completion of both steps in 15 min. US assistance (20 kHz and 450 W) proved to enhance both linked steps, which resulted in a considerable shortening of the overall analysis time (*i.e.*, 552.1 and 552.3 EPA methods require 1 and 2 h, respectively, only for derivatization, prior to analysis of these compounds in drinking water).

Research on metabolomic pathways of herbicides with the help of US for sample preparation has been of great interest to the agronomical field. Thus, studies on the metabolism of glyphosate in different plants such as *Lolium ssp* [67], *Clitoria ternatea* and *Neonotonia wightii* [68], and *Digitaria insularis* [69] have been monitored through herbicide decrease and formation of the corresponding metabolites after leaching all them with the assistance of US and separation of the target compounds by capillary

electrophoresis for final photometric detection. USAL and introduction of the leachate into the analytical equipment by the electroinjection mode were the key to obtain clean electropherograms, which allowed quantitation through the corresponding calibration curves from commercial standards of the target compounds.

Even more interesting are metabolomics studies on glufosinate [70] and imazamox [71] for whose metabolites there are not commercial standards. The metabolism of both herbicides in plants resistant and susceptible to these herbicides, which have also shown the influence of genetic modifications on the metabolic pathways of the herbicides, has recently been revealed. In both cases, acceleration of leaching was studied using MW or US, the former being less effective for removal of the target compounds and producing faster degradation of them; therefore, USAL was the advantageous selected approach. The aspect common to both studies was the absence of commercial standards of the corresponding metabolites, which forced to identify them by time-of-flight (TOF)/MS detection subsequent to LC separation. After characterization by this equipment, a simple method was proposed for semiquantitative determination of the metabolites based on LC–DAD and calibration curves from either the precursors or the commercial standard more similar to the given metabolite.

Also belonging to the xenometabolomics field is the research by Alcaide-Molina *et al.*, who studied the metabolites involved in the shikimate pathway (phenylalanine, tryptophan, tyrosine and shikimic acid) as biomarkers of the effect of glyphosate on *Triticum aestivum* exposed to this herbicide. The target metabolites were leached from wheat model plants with the help of US, which drastically reduced the time required for this step [72].

5.2. USAL of metabolites from animal and clinical samples

US has so far been scarcely applied to enhance leaching of target metabolites from animal or human samples. Some examples that show the advantages of application of this type of auxiliary energy, and can promote its use, are as follows:

A US device (40 kHz and 125 W) was used to accelerate leaching of bufadienolides such as bufalin, cinobufagin and resibufogenin from toad venom samples prior to individual separation–determination by HPLC–DAD. With this aim, 0.2 g of sample was mixed with 70:30 (v/v) methanol–water solution, then subjected to US (applied by an

ultrasonic bath for 20 min at 20 °C). A similar yield to that provided by USAL in 20 min required 6 h by SE, and 18 h by maceration [27].

6. US assisted steps in polymer matrices

The special action of US on polymer structures deserves to be considered; nevertheless, the number and variety of applications and the sometimes doubtful relationship of these steps with metabolomics make convenient condensation of the information as listed in Table 1. A brief comment of the most salient aspects to offer readers an overview of the advantages, disadvantages and no studied or no clarified aspects of US assisted steps in dealing with polymers is below.

The increased surface area of immobilized cellulase [73]; the favorable effect on glutarylation of sugarcane cellulose without catalyst requeriments and with an unclarified contribution of the presence of an ionic liquid [74]; the way by which the yield of polysaccharides from corn silk [75] or from pumkin [76] or that of lignans from *Shisandran chinensis* [77] is increased after exhaustive optimization by using the appropriate response surface design; the shortening of time and energy required for extraction of β-glucans from barley, of great interest for scaling up the process [78], all them show the accelerating effect of US, despite only in two of these studies [73,54] the effect of this energy on the structure of the target material has been analyzed.

The assertion of Milic *et al.* on the influence of US only on washing and no on diffusion of extractable resinoid substances from the aerial parts of white lady's bedstraw [79] deserves a more in depth research using both different US conditions, particularly frequency, and phenomenological models. Remarkable is the study on the effect of high power US to extract podophyllotoxin from *Podophyllum peltatum*. SEM analysis showed microscopic structure change on the tissue cells and increased pore volume and surface area after ultrasonic treatment, with a significant positive effect on the extraction yield [80]. Also the recent contribution by Yang *et al.* supports the drastic shortening of the process time required to extract baicalin and baicalein from *Radix Scutellariae* under the influence of US as compared with heat reflux extraction, with also a very significant reduction of temperature and solvent consumption; thus making the US assisted method very valuable for large scale implementation [81].

Table 1. Use of US to accelerate or enhance changes in polymer matrices.

Extracted compound	US device	Optimum working conditions	Extraction yield	Other features	Ref.
Free cellulose activity Immobilized cellulase activity	5 US probes (18, 20, 24, 26 and 29 kHz)	24 kHz and 15 W for 10 min 24 kHz and 60 W for 10 min	Increased activity: 18.17% Increased activity: 24.67%	Fluorescence, CD and SEM measurements of US effects	73
Glutarylation of sugarcane bagasse cellulose	Cleaning bath	40 kHz, 180 W, 20–120 min, 70–95 °C, ionic liquid	Substitution ranging from 0.22 to 1.20	Without catalyst. Structural characterization of glutarylated cellulose by FT-IR, and CP/MAS ^{13}C NMR	74
Polysaccharides from corn silk	With digital time and temperature controller	20 kHz, 250 W, 17 min, 56 °C, 1:20 g/mL solid–liquid(water) ratio	6.06%	Box–Behnken response surface design	75
Polysaccharides from pumpkin	With 2 cm flat tip probe	40 kHz, 70 W, 25 min, 70 °C, 1:10 g/mL solid–liquid (water) ratio	16.04±0.53%	Central composite rotatable response surface design	76
β-Glucans (>260 kDa) from barley	Processor with a 22 mm probe	24 kHz, 400 W, 17 min, 55 °C, 80% amplitude, 1% cycle, water	66.1%	Time and energy required *vs* stirred tank extraction: 3 min *vs* 3 h and 170 kJ/L *vs* 1460 kJ/L. Decrease of molecular weight with US application time.	78
Lignans from *Schisandra chinensis* seeds	Crasher with digital timer, power and temperature controller	40 kHz, 800 W, 61 min, 25 °C, 1:20 g/mL solid–liquid(19% ethanol, 25% $(NH_4)_2SO_4$, 56% water) ratio	13.10 mg/g schizandrin, 1.87 mg/g schisantherin A and 1.84 mg/g deoxyschizandrin	Use of 19% ethanol, 25% $(NH_4)_2SO_4$, 56% water instead of pure ethanol duplicated yield.	77
Resinoid from aerial parts of white lady's bedstraw	Cleaning bath	40 kHz, 120 W, 30 min, 40 °C, 50% aqueous ethanol	25.1 g/100 g	US influenced washing but not diffusion of extractable substances.	80
Podophyllotoxin from rhizomes of *Podophyllum peltatum*	Homogenizer connected to a sonotrode with 7 mm flat probe	24 kHz, 35 W, 10 min, 0.425–0.6 mm particle size, water as the medium	Brunauer–Emmett–Teller surface area : 1.1887 m^2/g, Langmuir surface area: 1.8542 m^2/g, cumulative volume of pores 0.003768 cm^3/g, average pore size 56.1844 Å	SEM studies showed changes in microscopic structure.	79
Baicalin and baicalein from *Radix Scutellariae*	Cleaning bath with temperature-controller	Common optimum values: 40 kHz, 180 W, 20 min, 0.355 mm particle size, 1:10 g/mL solid–liquid ratio, 3 cycles, pulse US mode Baicalin: 60 °C, 40% ethanol Baicalein: 30 °C with ethanol concentrations of 50% (v/v)	110.3167±4.1258 mg baicalin/100 g 37.4254±1.7665 mg baicalein/100 g	Drastic decrease of extraction time, temperature and solvent consumption as compared to the heat reflux method.	81

CD, circular dicroism; SEM, scanning electron microscopy; FT-IR, Fourier transform-infrared; CP/MAS ^{13}C NMR, cross polarization/magic angle spinning ^{13}C nuclear magnetic resonance.

7. US assisted liquid–liquid extraction

Despite US application favors mass transfer between immiscible phases —provided the involved partitioning equilibrium facilitates the transfer [82]— drastically shortens the extraction time, and enhances sensitivity as a result of the favorable effect of sample–extractant volumes ratio, few applications involving US, LLE and metabolomics appear in the bibliography on this subject. The high user involvement and the difficulty for automation could be the reasons for this situation.

Most authors working on USALLE use volumes within the range from few mL to few μL (which can be justified only either for expensive reagents or when the required preconcentration factor demands less extractant volume according to the partitioning factor and sample volume). In metabolomics, USALLE has been used mainly in discontinuous approaches and at conventional and micro scale [83]. Examples of USALLE of metabolites from plant and animal or clinical origin are discussed below.

7.1. US assisted liquid–liquid extraction of metabolites from plant origin samples

The sensitivity and repeatability of USAELLE and solid phase extraction (SPE) have been compared for preparation of samples of five commercial wines with markedly different aromatic characteristics and a synthetic wine, prior to the analysis of up to 44 volatile compounds. Many aroma compounds such as alcohols, esters and acids were common to all the samples, but some others no. The method providing higher recoveries was named as LLE-I (30 min sonication at 25 °C under a nitrogen atmosphere with diethyl ether-*n*-pentane mixture as extractant), which also allowed a greater number of aroma compounds to be extracted; while the SPE method presented higher repeatability. On the contrary the LLE-II method (15 min sonication at 25 °C with dichloromethane as extractant) was the least convenient for sample preparation prior to the analysis of the volatiles in the surveyed wines [84], thus showing the necessity for an exhaustive optimization of sample preparation.

7.2. US assisted liquid–liquid extraction of metabolites from samples of animal and clinical origin

Despite the also scant use of USALLE for metabolites from biological fluids, the

examples below show the potential of this technique also in this metabolomics area. It should be noted that the use of US accelerates the LLE step, but is does not avoid the need for previous steps mandatory to remove proteins and other high-molecular weight components of biofluids.

Sample preparation of human urine prior to determination of three strogens (17β-estradiol, estrone, and diethylstilbestrol) required protein precipitation by inorganic salts such as $ZnSO_4$, $MgSO_4$, and $PbAc_2$ and subsequent centrifugation. Then, a US assisted cloud point extraction method was based on the induction of an organized micellar medium by using a no ionic biodegradable surfactant (Tergitol TMN-6) to extract the strogens using 10% (v/v) of Tergitol TMN-6 solution and the help of a thermostated ultrasonic bath acting for 45 min at 35 kHz. Finally, the target metabolites were determined by HPLC–DAD [19].

A more interesting approach was used for sample preparation prior to the simultaneous determination of quinocetone and three of its desoxy metabolites in swine urine. US-assisted dispersive liquid–liquid microextraction was carried out using tetrachloromethane in acetone as extractant and an ultrasonic water bath for 3 min to form an emulsion with subsequent disruption by centrifugation. The target metabolites were both in the solid and the extract at the bottom of the test tube; therefore, the aqueous solution was discarded and the rest was used for analysis (after dissolving the remaining droplet and lipidic solid in acetonitrile) based on individual separation by HPLC and determination by a coupled variable wavelength detector [20].

A complex approach was used for determination of five phenols in human urine and blood samples, because prior to extraction of the target analytes by US assisted emulsification, microextraction and solidification of the floating organic droplet, a step to remove proteins and other high weight molecules was mandatory. It consisted of mixing 2 mL of the target human fluid (serum or urine) with acetonitrile at 1:2 ratio, subsequent centrifugation for 3 min and adjustment of both the ionic strength and pH of the liquid phase [85]. A subsequent multi US-assisted step involving simultaneous US assisted emulsion formation and US assisted LLE —*viz.*, the socalled US assisted emulsification LLE [31]— was developed by using 10 mL of the prepared sample and 40 μL of 2-dodecanol under application of US (40 kHz for 5 min at 45 °C). The micro droplet of the extract, solidified in an ice bath, was collected with a spatula and finally subjected to CE separation. These sample preparation methods require high user involvement but simple analytical tools.

8. US assisted (bio)chemical reactions in metabolomics

In the light of the research so far developed in dealing with metabolomics studies in which reactions have been improved by US application, this energy has been mainly used to enhance hydrolysis of metabolites as sialic acids (SIAs) and enzymatic hydrolysis (using mainly β-glucuronidase) in clinical samples.

8.1. US assisted hydrolysis reactions in metabolomics

Hydrolysis of conjugated metabolites in clinical samples is a slow step drastically accelerated by US. Some examples support this assertion.

A method for determination of SIAs shows how a US device can be used to assist different steps of the analytical process by the semiautomatic platform shown in Fig. 2. The method, applied to serum, urine, saliva and breast milk samples, consists of the following steps: hydrolysis of SIA conjugated derivatives, SPE and derivatization as sample preparation prior to insertion of the analytical sample into either a μLC–laser induced fluorescence detector or an LC–MS/MS triple quadrupole mass detector [86]. Deconjugation of SIAs was carried out by sample acidification and immersion of the flask containing the sample into a water bath at 40 °C, where ultrasonic energy under the optimal working conditions —0.5 s/s duty cycle, output amplitude 40% of the converter (180 W) and sonication time for 20 min— was applied. The released SIAs were then efficiently concentrated in a dynamic manner using a lab-on-valve module that allows automation of SPE for preconcentration and cleanup. In line with SPE derivatization of SIAs by DMB labelling was carried, thus shortening the time from 180 min, required by the conventional heating method, to 20 min when assisted by US.

On the other hand, Álvarez-Sánchez *et al.* [23] optimized a sample preparation protocol to obtain a metabolite profile of human saliva by LC–TOF/MS in high accuracy mode. Acidic and mild basic hydrolysis conditions were tested for sonicated (50% of total output amplitude, 0.5 s/s duty cycle and 10 min US application) and no sonicated samples. US proved to enhance and accelerate hydrolysis with an associated effect of increased metabolite coverage. While US energy did not have any impact on the number of detected features in negative ionization mode, a considerably increase of detected features in both acidic and basic hydrolyses was found in positive mode. Thus, US-assisted hydrolysis led to 38 unique features in basic hydrolysis, in contrast to 16 entities obtained by hydrolysis

without US assistance. Amino acids, sugars, lipids antioxidants and other potentially interesting biomarkers such as polyamines, vitamin B3, and ethylphosphate were identified. This was the first time US was involved in saliva sample preparation.

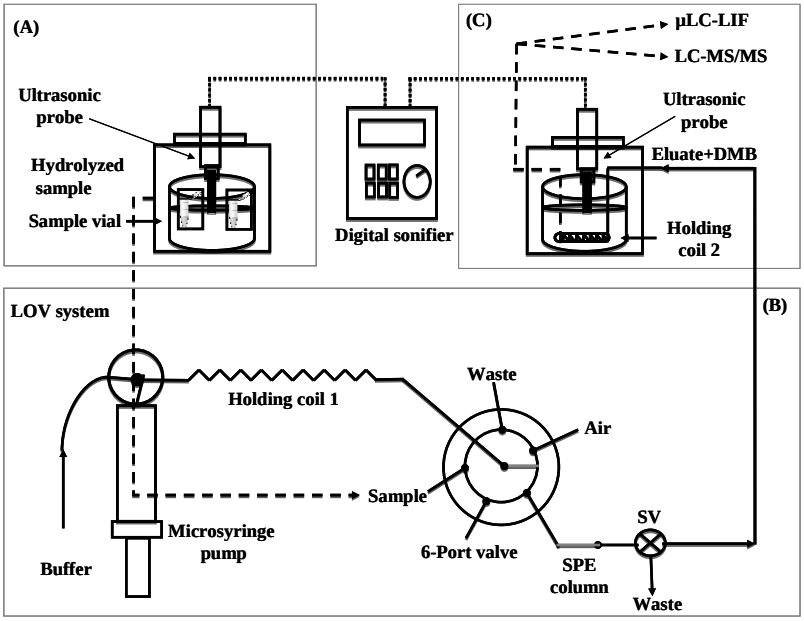

Fig. 2. Experimental scheme for sample preparation: **(A)** and **(C)** ultrasound-assisted hydrolysis and derivatization, respectively. Dashed line, no automated process; dotted line, exchangeable position of the probe for derivatization. **(B)** LOV system for SPE. Continuous line, automated process. DMB, derivatizing reagent; LC–MS/MS, liquid chromatograph with triple quad mass detector; µLC–LIF, microliquid chromatograph with laser induced fluorescence detector; SPE, solid phase extraction; SV, switching valve. (With permission of Elsevier, Ref. [86]).

Also an overall method has been proposed for targeted quantitative analysis of aliphatic alcohols, triterpenes and phytosterols in human serum in which saponification and derivatization were accelerated by US assistance. The method started by protein precipitation; then, saponification consisted of addition to the deproteinated sample of methanol 2 M KOH and immersion of the reaction mixture vessel into a water bath at 20 °C, where US —0.45 s/s duty cycle, 50% output amplitude of the converter— was applied for 10 min. After cooling at room temperature, the immiscible organic phase was separated by centrifugation and the unsaponifiable fraction was extracted with *n*-hexane and

subsequent treated with pyridine, BSTFA and TMCS, the mixture was shaken and then, subjected to US (50 s/s duty cycle, 40% output amplitude of the converter) for 10 min to accelerate the derivatization reaction prior to GC–MS analysis of the target metabolites. US allowed shortening of the sample preparation <35 min for the organic phase and <48 min for the unsaponificable phase. The total analysis time was 71 min, and 10 min extra time was necessary for re-establishing and equilibrating the initial conditions [87]. The advantage of using the same US probe to assist both steps, thus decreasing the time required by their development, should be counterbalanced by optimizing the US frequency, which could give better results to the detriment of simplicity of using a single US device, if the optimum values of this variable were different for each of the given steps.

Sequential derivatization of esterified fatty acids (EFAs) and no esterified fatty acids (NEFAs) for independent GC–MS analysis of both fractions of metabolites can also be favored by US. Human serum from obese individuals was the sample subjected to the US assisted step under the optimum working conditions; first in a basic medium for EFAs, and then in an acidic medium for NEFAs. The time required for methylation was 5 and 15 min, for EFAs and NEFAs, respectively, drastically shorter than for no sonicated samples [88].

8.2. US assisted enzymatic reactions in metabolomics

US has so far been scarcely applied to enhance enzymatic reactions, despite the advantages involved in application of this type of auxiliary energy have been demonstrated.

The analysis of female steroid hormones, which exist in urine as conjugated forms (mainly, glucuronides and sulfates), requires deconjugation by enzymatic hydrolysis (usually by β-glucuronidase with sulfatase activity). This slow step can be drastically accelerated by US energy in such as way that, under the optimum US conditions, it is completed within 30 min *versus* 12–18 h required for no ultrasonicated samples [89].

An interesting approach for sample preparation prior to determination of the sunscreen agent 2-ethylhexyl 4-(*N,N*-dimethylamino)benzoate (EDP) and its metabolites in human urine by automated on line SPE–LC–MS/MS with LC–TOF/MS confirmation was reported by León-González *et al.* [90]. Determination of both free and conjugated forms of the target metabolites required two urine aliquots, one of them in the absence of the biocatalyst and the other with addition of β-glucuronidase solution and subjected to US

energy for 50 min at 20 kHz, at 35% duty cycle and 50% output amplitude (converted applied power, 400 W)] to deconjugate any phase II EDP metabolites potentially existing in the urine. Similar sample preparation required the determination of EDP and its metabolites in human semen also by automated online SPE–LC–MS/MS [91].

Galesto *et al.* reported an in depth study on the enzymatic hydrolysis of anabolic androgenic steroids excreted to urine as glucuronide conjugates also improved by US energy [92]. The time required for hydrolysis catalyzed by β-glucuronidase from *E. coli* K12 was shortened by a factor of six when US was applied. The authors concluded that US enhances β-glucuronidase enzymatic kinetics by increasing both initial velocity and maximum velocity and that the improvement caused by this energy is mainly due to both stabilization of the transition state and enhancement of the mass transfer processes. Assessment of these conclusions is a pending goal for the authors or other groups working in this area.

9. Conclusions

In the light of the research so far developed in the field of metabolomics, it is clear that the use of US for the steps involved in sample preparation and other preliminary steps should be promoted. It is remarkable that US application always shorten the time required for extraction of metabolites from h (by conventional methods) to min, which save energy and costs.

It is worth to emphasizing that US energy also causes undesirable effects, mainly degradation, which should be widely studied with three main aims: (i) to know the nature of the degradation products, and thus the pathways through which degradation occurs; (ii) to prove the influence of the free radicals formed by influence of cavitation; (iii) to study the effect of US frequency on the degradation processes by the decrease of cavitation taking place by gradual change of this variable from power to high frequencies. Despite the benefits of automated frequency scanning demonstrated by Romdhane and Gourdan [93], later ratified by Vilkhu *et al.* [94], no subsequent studies in this direction have been developed nor appropriate equipment for their implementation has been commercialized. In fact, most of the authors work at fixed frequency (usually 20 or 40 kHz), they do not include the frequency within the characteristics of the US device, and do not develop studies on possible degradation or changes induced for US application.

It is also remarkable that US has been widely used to improve steps of a wide number of plant origin samples, but this energy has been scarcely applied to enhance steps of target metabolites from animal or human samples, possibly due to the fact that clinical personnel are unfamiliar with US. In addition, US has been used more in targeted than in untargeted analysis (despite the fact that the digestion assisted by US possibly degrades less than helped by heat —aspect that should be checked). USAL at different US frequencies could provide greater selectivity for compounds families and, therefore, be of great interest in targeted analysis.

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Chapter III

A review on enzymes and ultrasound: A controversial but fruitful relationship

A review on enzymes and ultrasound: A controversial but fruitful relationship

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A review on enzymes and ultrasound: A controversial but fruitful relationship

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Abstract

A critical review on the effect of ultrasound (US) on enzymes and their biocatalytic action is presented here. Discussion on the information users of US acquire before utilizing the different devices, and the importance they give to US frequency is constant along the review. The authors have gone into the different areas in which the US–enzyme binomial has been applied. The lack of enough information on the US–enzyme–working conditions under which each piece of research has been developed, and the necessity to provide complete information on the data and metadata to give enough light on each piece of research (and thus on the potential comparison of results from different studies) are critically exposed. With this aim, the study has been divided into the positive effect of US on enzymes to favor the production of metabolites, polymers or proteins; and the degradation, inhibition or activation of the biocatalyst under US application. Also the effect of US on enzymes production and the main fields of application of the US–enzyme binomial are discussed.

Keywords: enzymes, ultrasound, metabolites, proteins, polymers, enzymatic activation/inhibition.

1. Introduction

Ultrasound (US) is a type of no radiant energy (therefore, it is incorrect to express its application as "US irradiation") each time more used by analytical chemists despite the knowledge some of them show about it has not grown at the same rhythm as its use. Cavitation, the most named phenomenon produced by US [1], is highly dependent on the frequency of US (property scantly considered by some of its users, even sometimes forgotten when US equipment is described). The cavitation phenomenon contributes to the US effect on enzymes through three main mechanisms, the effect of which is considered to act separately or combined, by either improving enzyme catalysis or degrading the biocatalyst. One of the effects is purely thermal, due to the enormous microzone temperatures achieved during cavitation. Other effect is due to free radicals generated by ultrasonolysis in either water or other polar liquids. The third effect is caused by the mechanical forces, shear forces, created by microstreaming and shock.

Present literature regarding the time US is applied to enzymatic reactions can be categorized into two main groups: the first makes use of US sample preparation prior to the enzymatic step [2]. In this case, reduction in particle size and a consequent increase in the catalytic surface area are useful to reduce mass transfer limitations. The second approach involves the use of US throughout the enzymatic reaction. Here, the cavitation energy is thought to accelerate the reaction rate, yet the mechanism by which this occurs is unclear (perhaps by increasing the movement of liquid molecules, the substrate's access to the active site is increased). The second approach has demonstrated either to accelerate enzymatic reactions or to inactivate the biocatalyst [2,3], but in the former case with a dependence of the enzymatic enhancement with the intensity of ultrasonication rather than with its frequency [4]. Apparently, cavitation promotes an increment in reaction rates rather than a change in the reaction constants.

In old literature, authors working with US have attributed its action to different effects. This is the case with acceleration of enzymatic hydrolysis, which is mainly caused, in the Tuulmets and Paik's opinion [5], by temperature gradients in the cavitation medium; while Vulfson *et al.* did not attribute to cavitation the effect of power US of 25 kHz (frequency no specified in the publication) on interesterification, which is also a hydrolytic reaction [6]. In dealing with enzyme degradation, the influence of moderate pressures on the system subjected to US is considered either to exert an enormous effect [7], or to cause little effect on enzyme degradation [8]. The main causes of controversial results can be

attributed to the different US frequencies, intensities and application conditions (no specified in the publication), and/or to the different types of enzymes on which US energy is applied and even to the different working conditions.

This review article is mainly aimed at showing, (i) the different areas in which the US–enzyme binomial has been applied, (ii) the lack of enough information provided on the US–enzyme–working conditions under which each piece of research has been developed, and (iii) the necessity for providing complete information on the data and metadata to give enough light on each piece of research and thus, on the potential comparison of results from different studies. Advances in this field are closely related to the possibility of comparing the results obtained by different authors under different/similar but well-known working conditions. Division of the review as a function of substrates instead of biocatalysts was decided both for a better interpretation of the influence of these components on the effects caused by the US–enzyme binomial and the importance of the products obtained as a result of the given process.

2. Ultrasound frequency and power/intensity, ultrasonic devices and working modes

This section deals with aspects that should be always included in any publication involving the use of US, but most times forgotten or scarcely explained.

2.1. Types of ultrasonic devices

Despite the well-known deficiencies of US cleaning baths when used for analytical tasks, for which they have not been designed [9], a number of researchers uses this device, omnipresent in analytical laboratories. The no reproducible performance of US cleaning baths and the decline of power with the working time are not taken into account by the authors, who do not consider these aspects affect the results. For example, these deficiencies are not reflected in the results obtained for arsenic speciation analysis in seafood when the sample, after US-assisted enzymatic hydrolysis, was subjected to solid-phase extraction (SPE), HPLC separation and ICP–MS detection [10]. It is worth emphasizing the growing number of analytical chemists who use US probes or reactors designed for specific uses, as commented below. The inappropriate name of “instrument”

given by some authors to US probes [11] must be avoided as these devices never provide analytical information.

2.2. Ultrasound frequency and power or intensity

As the name implies, the frequency of US is beyond the sound frequency; nevertheless, the prefix "sono" and the adjective "acoustic" are used in dealing with US (*e.g.*, sonochemistry is a term used in US application on chemistry). This error should be avoided as the US range encompasses from 20 kHz (human hearing reaches up to 16–18 kHz) to GHz, with division between power US (20–100 kHz) within which cavitation is a predominant force, and diagnostic US (5 MHz–GHz) [9]. A "sonochemistry range" is considered to encompass from 20 kHz to 2 MHz, in which the zone within or close to MHz is considered as therapeutic. This last zone has been scarcely used by analytical chemists, but some pioneer research using it deserves to be pointed out: such is the case with the study, in 1975, on conformational changes produced by 880 kHz US on penicillin amidase (monitored by pH changes and assisted by a pH-Stat) [12]; or that developed in 1985 by Chetverikova *et al.* about the *in vitro* effect of US of the same frequency on different enzymes (kinases and dehydrogenases) as a way to detect if this primary US-biological interaction could be extrapolated at a higher level of organization complexity. They concluded the presence of minor effects that were not investigated using lower frequencies [13]. *In vivo* studies in rats developed about 15 years later on inhibition of enzymes of drug metabolism in the liver by US (then corrected by heparin), only included the intensity and duration of US application (2 W/cm^2 for 1 min) [14]. A purely analytical study dealt with the effect of therapeutic US (815 kHz) on the hydrolysis reaction of sucrose catalyzed by invertase, in which significant enhancement of enzyme activity was obtained, especially at low sucrose concentration [15].

Apart from these few cases of specific use of US within the therapeutic range, most of the studies involving enzyme–US can be divided into six groups, in a growing order attending to the information provided on the characteristics of the US device used:

(i) *Without information*. In some cases no information at all is given about the US device used. Maybe the lack of information on the US device and US variables was the reason why reproduction of the results obtained by ultrasonication of lipase from porcine pancreas (up to 83% enhancement) has been hindered for years [16].

(ii) *With information on the model of the device*. The scant information provided in a number of publications —data such as "a model Transsonic TI-H-5 ultrasonic bath from Elma (Singen, Germany), a model UTR200 cup horn sonoreactor (SR), and a model UP 50H (UP) ultrasonic cell disruptor, both from Dr. Hielscher (Teltow, Germany)" [17], a UP200 S Hielscher, 200 W ultrasound equipment operating at 23 kHz, and 20% to 100% of the total power [18], or "an ultrasonic bath (KH2200B, Herchuang Co., Kunshan, China)" [19]— should not be allowed as the only information about the devices used.

(iii) *With information only about US power and/or intensity applied*. Information such as "US bath of 60 W" [20], "water bath supply 125 W and tank capacity 2.8 L" [21], "power of ultrasound irradiation (50, 100, and 120 W)" [22], or "ultrasonic-assisted enzymatic hydrolysis at specific ultrasound power (typically, 100 W) for a given time" [23], seem not to be the proper information a researcher needs when trying to reproduce an experiment.

(iv) *With information about frequency*. Information on this US variable should be mandatory, but not sufficient, in any publication on the use of this type of energy [24], with welcome data about other variables, either related to US such as duty cycle [25] or physical variables that US action can modify, as temperature [26]. Especial care should be devoted to variable units, without confusion between US power (W) and US intensity (W/surface unit, usually cm^2) [27].

(v) *With enough information about the characteristics of commercial conventional US devices*. Distinction should be made between devices providing single or multiple frequency of US. In the former case, the most common information is frequency and either, power or intensity for probes [28,29] or baths [30,31]. Additional information on the surface area of the US transducer (usually fitted at the bottom) is sometimes given in dealing with US baths [32]. A debatable concept is to attribute to probes a "focused" action, reflected even in the title [33]. In fact, there is not a guide for US in the probe; therefore, the effect is not focused, as is the case with wave-guides in some microwave devices [34]. It could be said that a US probe restricts its action zone with respect to a bath, but never focused US. In some research using both a bath and probe, information on frequency and power of the bath is given, but only the model in the case of the probe [35]. Information about both devices makes comparison easier for readers.

An odd frequency (423 kHz, far from the audio frequency) of the device named as "sonoreactor" has been profusely used for collaborative research between groups [36–38].

Multifrequency US devices encompass either narrow or wide range of frequencies. Examples of the former are a device that works at 18, 20, 24, 26, and 29 kHz, with different probes [39], and other working at 16 and 20 kHz [40]. Little or no differences were found in these cases for the different frequencies of US when working at the same power. Higher differences are found when the US range is wider, as is the case with an ultrasonic bath working at 40, 80 and 100 kHz, and powers of 100, 150 and 200 W, with the best effects on transesterification at 80 kHz and 150 W [41]: a US zone with chemical effects as opposite to the mechanical effects, the latter being the main effects of lower frequencies. Two baths, each endowed with two frequencies (28/40 and 50/100 kHz), have been used with optimum action on the biocatalyzed system at 40 kHz and intensity of 0.5 W/cm^2 [42]. The different substrates, enzymes and expression of US variables make comparison of the different studies unfeasible.

Performance comparison of probes *versus* baths working at different frequencies and powers can only be based on the results they provide when acting on a given enzyme–substrate system [43,44].

(vi) *With information about no conventional US devices*. Application of heat and US under moderate pressure has been carried out by López *et al.* [7] using a device designed by the authors and called by them as a thermoresistometer [45]. Also a curved US transducer was devised by Oulahal *et al.* to standardize biofilm removal for hygiene testing in internal or curved food contact surfaces [46]. Other no conventional and no commercial transducers have been described elsewhere [9].

Mention apart deserves the discontinuous application of US that some authors designs as "pulse" and that should be designed as duty cycle. To express this parameter as percent is also incorrect as no information is given about how long each US application lasts. The correct way is as the number of times units of application per total units of the cycle (*e.g.,* 5 s/9 s) or as a fraction of the unit of time that US application lasts (*e.g.,* 0.5 s/s).

3. Behavior of enzymes when subjected to US application

3.1. The influence of the reaction medium on the effects of US on enzymes

It is a proved fact that enzyme performance is greatly affected by the nature of the reaction medium [47]. This effect is enhanced in the presence of US, but it has not been studied and/or discussed in detail by researchers. Especial mention deserves the use of ionic liquids (ILs) in the reaction medium because their characteristics dramatically influence the US–enzyme behavior most times.

The effect of ILs on the isomerization of glucose to fructose by glucose isomerase was investigated by Wang *et al.* [48] using 12 imidazolium ILs with different cations and anions in the presence and absence of different organic solvents. The authors demonstrated that the enzyme activity was greatly dependent on the properties of both the ILs and organic solvents. In most of the cases, higher enzyme activities were observed in ILs as compared with those in solvent free or organic solvent media. The enhancement of enzyme activity in ILs as compared to that in organic solvents might be caused by the highest capability of ILs to dissolve substrates (the glucose solubility in most ILs is much higher than in organic solvents), which enables the reaction to develop in homogeneous media [49]. The different reactivity patterns observed in different ILs (decreased by decreasing the length of the IL alkyl chain for the same anion: decreased hydrophobicity) is similar to that of the screened organic solvents, and in line with the literature: the enzymes usually exhibit good performance in hydrophobic solvents because hydrophilic solvents might disrupt the functional structure of enzyme by stripping off the essential water, whose function was to preserve the correct molecular conformation of the enzyme [50]. US application in these media plays a dual role: US decreases the high viscosity of the IL and increases interaction change between the substrate and the enzyme in the reaction medium [51]; but US would also inactivate the enzyme. The negative effect of cavitation on enzyme activity could be avoided or minimized by immobilizing the biocatalyst, the silica coating protecting from this effect.

3.2. Behavior of free and immobilized enzymes under US application

Most enzymes involved in the biocatalyst–US binomial have been hydrolytic

enzymes (particularly lipases, amylases or proteases), either as simple biocatalysts or in complex mixtures. Both in solution and immobilized enzymes are commercially available from Sigma–Aldrich, Novozymes and other suppliers, but also in-laboratory immobilization has been carried out, either individual or in mixture, as is the case with cellulose, pectinase and hemicellulose for which alginate was used as support and glutaraldehyde as the coupling agent [52].

Despite the action of US can significantly decrease the activation energy of enzymes, this type of energy can also alter the behavior of enzymes that respond differently to alterations of pH and temperature in the presence and absence of this type of energy, as is the case of α-amylase and amyloglucosidase [53]. While the different response to pH is not explained by the authors, the influence of temperature is explained by both an increase in activity by the increase in temperature in the presence of US, and the decrease of this effect by inactivation of the enzymes favored by US at temperatures higher than 50 ºC.

Comparison of the impact of US on free and immobilized cellulase showed that the highest activity of the free biocatalyst was achieved when the sample was treated with 24 kHz US at 15 W, for 10 min, under which the enzyme activity was increased by 18.17% over the control. Fluorescence and circular dichroism (CD) spectra revealed that the ultrasonic treatment had increased slightly the number of tryptophan on the cellulase surface, with partial deformation of the α-helix structure and an increase of random coil content in cellulase protein. The highest activity of immobilized cellulase was achieved when the sample was treated with 24 kHz US at 60 W for 10 min, under which the activity of the enzyme was increased by 24.67% over the control. Scanning electron microscopy (SEM) revealed that the ultrasonic treatment had increased the surface area of the immobilized cellulase [39].

Concerning reusability of immobilized enzymes, the increase in activity of commercially available lipase immobilized on beads of a macroporous acrylic resin when subjected to low intensity US was kept after being used up to 4 times [54], and even up to 8 [55] or 10 times [56].

Also US can act on the support for enzyme immobilization improving its characteristics by controlling the morphology of mesoporous silica without additives. Discrete rod-like and string-of-beads like mesoporous SBA-15 can be achieved by US application (45 kHz, 400 W) without changing the composition of the synthesis system.

The smaller particles of SBA-15 showed to improve both lysozyme immobilization capacity and adsorption rate over conventional rope-like SBA-15 [57].

3.3. Influence of US variables on enzymes

It is worth emphasizing that comparison of research developed with different US equipment acting on enzymes is not conclusive (especially when US is applied on different enzyme–substrate systems and in different media); nevertheless, a common behavior is inactivation of the enzyme in some proportion, an effect that increases by increasing power or intensity, but can be differently affected by US frequency.

3.3.1. Influence of US frequency

Comparison of different but close *US* frequencies (20-to-40 kHz) applied by the same US device to the same immobilized enzyme acting on the esterification of two different substrates, rutin and naringin, shows the same optimum value for frequency in both cases (25 kHz), but different optimum US power (200 and 150 W for rutin and naringin, respectively) [55]. On the contrary, comparison of the effect of different US frequencies by using different devices on the same enzymatic process does not provide conclusive results. This is the case with the use of a probe (50 W, 30 kHz) and a reactor (200 W, 24 kHz), both working at 50% amplitude, to accelerate proteins digestion. The use of cutting-edge analytical equipment (MALDI–TOF–MS) to analyze the digested sample provided as only conclusion that a dirtier digested sample was obtained by using the probe as a consequence of more direct US application [43].

3.3.2. Influence of US power

A study on the use of a commercial lipase to produce diacylglycerol by hydrolysis of palm oil under US application ("ultrasound equipment" of 200 W at 23 kHz, and 20% to 100% of the total power) showed that exposition of the reaction system to US led to enzyme inactivation and water evaporation [18]. On the contrary, when US was used to promote emulsification of the water–oil system before the hydrolysis reaction, thus avoiding contact between the probe and the enzyme, an increase of the reaction product was obtained in a shorter time, as shown in Fig. 1. A more recent study, also on hydrolysis of triglycerides at

different powers (45, 85, 125, 165 and 200 W) and duty cycles (20%, 40%, 66% and 80%, duration no expressed), and fixed frequency of 25 kHz of an ultrasonic bath, was carried out to know the influence of these variables on the reaction rate of dairy waste-water pretreatment. A linear increase of hydrolysis from 45 to 165 W was found and the highest hydrolysis of about 78% was obtained at 165 W at a time span of 40 min. At high power, a large number of bubbles was formed that collapsed violently. Concerning the duty cycle, the hydrolysis increased with the increase in duty cycle from 20% to 66%; then, it remained consistent at 80% duty cycle (not specified duration). The increase in the hydrolysis was explained by the fact that when the time of exposure to US increases bubble formation, implosion also increases, and the reaction rate as a result. Nevertheless, long US application times were not recommended owing to excessive heating, which denatured the enzyme and created problems to transducers [58].

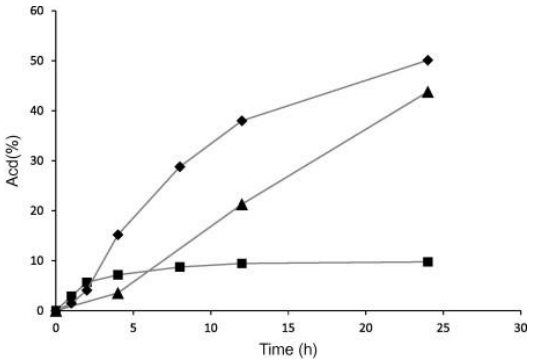

Fig. 1. Free fatty acid content as a function of the reaction time. ■, Reaction assisted by US for the whole reaction time; ♦, two-step reaction (using US to promote emulsification before the reaction); ▲, control reaction (without US assistance). (With permission of Elsevier, Ref. 18).

The effect of US power on cellulase showed a high dependence of its activity on this US variable, which was more pronounced in the presence of a US reflector. Without a reflector at 40% amplitude of the probe (40 kHz, 500 W of maximum power), a 12% loss of enzyme activity was measured in a period of 65 min. By increasing the amplitude to 60 and 80%, the decrease in enzyme activity became more significant, and after 65 min the enzyme possessed only 80 and 75% of the original activity, respectively. Despite the applied ultrasonication depressed the activity of cellulase, the outcome of the enzyme catalyzed

hydrolysis in the reaction model was always positive; therefore, the advantageous effects of ultrasonication on the heterogeneous cellulose–cellulase reaction always overcome the undesirable effect of US on enzyme modification [59].

3.3.3. Effect of US intensity

A recent study deals with the effect of low intensity US application on the kinetic and thermodynamic parameters as well as on the molecular structure of cellulase. With this aim, the chemical reaction kinetics model, Arrhenius equation, Eyring transition state theory, Michaelis–Menten equation, fluorescence spectrometry and CD spectroscopy were used. The maximum cellulase activity was observed at 17.33 W/cm^2 intensity and ultrasonic treatment for 30 min, under which the enzyme activity was increased by about 25% over the untreated enzyme. After US treatment, the typical thermodynamic parameters were all reduced, as did the number of tryptophan on cellulase surface, and the molecular structure of cellulase changed favorably to provide more access to the active sites [60].

A previous study, developed more than 15 years ago and, therefore, with no cutting-edge instrumentation for monitoring, was aimed at knowing the effects of 20 kHz US at different intensities (46, 92 and 138 W/cm^2) on the catalytic activity and structure of the tetramer of wild-type human butyrylcholinesterase (BChE) from plasma and recombinant D70G mutant enzyme, working at constant temperature. The US effects were investigated using a neutral substrate (*o*-nitrophenylbutyrate), a positively charged substrate (butyrylthiocholine), and a negatively charged substrate (aspirin), and monitored by gel electrophoresis and photometric measurements of V_{max} after US treatment. For both wild-type and D70G biocatalysts, K_M increased slightly for butyrylthiocholine and *o*-nitrophenylbutyrate under US application, suggesting that the US effects were not due to the periodic variation of pressure but rather to shear forces that took off substrate from the peripheral site and altered diffusion to the active site. By contrast, affinity of the D70G mutant for aspirin slightly increased by increasing US power, suggesting that US-induced microstreaming unmasked peripheral residues involved in recognition and initial binding of the negatively charged substrate. Short US duty cycles induced a faint transient enzyme activation, but prolonged US application caused partial dissociation of the tetrameric enzyme and irreversible inactivation [8].

3.3.4. A common use of US in biochemistry: cell disruption

Cell disruption to extract enzymes is a common practice in biochemistry research, but the influence of the frequency of the US probe had not been comparatively studied. The results obtained from sludge flocs using US probes working at 20 and 40 kHz at different intensities showed that the former frequency allows extraction of more types of enzymes than the latter. Under the optimum conditions, US can extract extracellular enzymes and a small part of intercellular enzymes, being US intensity a more influential variable than US duration [44].

4. Metabolites as substrates of US-assisted enzymatic catalysis

The types of metabolites and enzymatic reactions that can be favorably influenced by US are numerous; therefore, the most outstanding processes involving these low-molecular compounds are discussed in this section.

4.1. US-assisted enzymatic hydrolysis

The type of reaction more widely assisted by the US–enzyme binomial in dealing with metabolites is hydrolysis. The following subsections are devoted to representative types of metabolites the hydrolysis of which has been favored by the given binomial.

4.1.1. Fats hydrolysis

Hydrolysis is the main first reaction for production of free fatty acids that may then be esterified, interesterified, transesterified or converted into high-value fatty alcohols. As compared with conventional methods for oil hydrolysis, enzymatic hydrolysis can be performed under relatively milder and simpler process conditions, and enhanced by US energy, either in solvent-free media or in heterogeneous media. Free lipases from different strains and immobilized on different supports are commercially available for this task. A review on this subject has recently been published by Lerin *et al.* [61].

Comparative studies on lipase-catalyzed hydrolysis of soy oil in solvent-free systems were carried out in a shaking bath US bath (ultrasonic intensity 1.64 W/cm^2 and frequency of 28 kHz). Under the optimum conditions, the overall enzymatic hydrolysis

assisted by US was 2 times higher than that assisted by shaking [31]. When the solvent-free US-assisted process of lipase-catalyzed hydrolysis of soy oil was compared with that taking place in a heterogeneous medium involving water, the latter was the most favorable situation as the US energy dispersed soy oil in water to obtain a larger interfacial area, which caused a higher initial rate of soy oil hydrolysis as compared with the solvent-free system [62].

Diacylglycerols (DAGs) are glycerol esters in which two hydroxyl groups are esterified with fatty acids that occur as natural components of glycerides in various fats and oils at levels up to 10% (w/w) [63]. The properties attributed to DAG oils as suppressors of serum levels of postprandial triglycerides [64], and as emulsifiers and stabilizers in foods, cosmetics and pharmaceutical industries have promoted their US-assisted production by enhancing the action of lipases, either in solution or immobilized. Solvent-free systems, with [35] and without the presence of surfactants [65], have been studied, mainly using commercially immobilized enzymes. Despite most of the studies on DAGs have involved the joint use of both immobilized enzymes and US, the previous application of this energy to promote emulsification of a water/oil system before the hydrolysis reaction avoids contact between the probe and the enzymes and provides excellent results [18].

Degumming is the first step in the refining process of vegetable oils, which removes phospholipids and mucilaginous gums by phospholipases. Most of the reported studies have so far been focused on the degumming efficiency of phospholipase A, the action of which has intended to be accelerated by the presence of US [66]. Despite the increased acceleration caused by US, the well-known effect of this energy on oils oxidation [67] advisers against application of US on edible oils.

An uncommon and recently developed reaction involving US–enzyme action was the enzymatic pre-hydrolysis of a synthetic dairy wastewater with around 2000 mg/L of fat content. Exhaustive optimization of parameters such as enzyme loading, temperature, US power, frequency, duty cycle and speed of agitation led to a maximum hydrolysis of 78% at 0.2% (w/v) enzyme loading, 30 ºC temperature, 165 W of US power at 25 kHz and 66% duty cycle (with not information on cycle duration) [68].

4.1.2. Hydrolysis of glucuronide conjugates

A number of liposoluble metabolites is excreted in urine after formation in the

organism of more hydrophilic glucuronide conjugates. The study of these metabolites makes mandatory a previous hydrolysis using β-glucuronidase, the biocatalysis of which is relatively slow. One of the first contributions to the use of US–enzyme in this field dealt with six female steroid hormones, found in woman urine as conjugated forms (mainly, glucuronides and sulfates). The free steroid forms of these metabolites were obtained by enzymatic hydrolysis using β-glucuronidase with sulfatase activity, and assisted by US energy (applied by a probe working at a frequency of 20 kHz, 225 W and 0.4 s/s duty cycle). A drastic shortening of the time required for this step as compared with conventional protocols (from 12–18 h to 30 min) was thus achieved [69]. Xenobiotics such as anabolic steroids in urine have also been deconjugated with the help of β-glucuronidase and a US probe in only 10 min. The different source of the enzyme, different metabolites and different US probe (no information but the commercial name was given) hinder comparison between the results in [69] and those obtained in this case [17]. A subsequent study on the same enzyme aimed at revealing the effect of US energy on the enzymatic hydrolysis kinetic parameters, as well as on the enzymatic activity of the biocatalyst was conducted using the compounds 4-nitrophenyl-β-D-glucuronide and 4-nitrophenol (product of the enzymatic reaction together with glucuronic acid). The experimental data suggested that the reaction follows the Michaelis–Menten kinetics and that US affects the initial reaction rate, which was higher when this energy was used and compared to the classical method of incubation at 55 ºC. Also the values of V_{max} and k_{cat} were higher for the ultrasonic assay, whilst the Michaelis–Menten constant obtained by the two methodologies showed similar values. Deactivation of the enzyme was also observed under US energy, which was found particularly evident for experimental conditions with excess of substrate. Key information on enzyme deactivation was not obtained because the simple analytical instrumentation used (HPLC–molecular absorption detector) [70].

The *in vivo* metabolism of a xenobiotic, a UV filter commonly used in sunscreen cosmetic products [2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate (EDP)], was studied by targeting metabolomics analysis in human urine. The metabolomic study involved the use of urine from male and female volunteers before and after application of an EDP-containing sunscreen cosmetic with and without enzymatic hydrolysis. The detected metabolites were subsequently confirmed by liquid chromatography–time-of-flight/mass spectrometry (LC–TOF/MS). The existence of phase II metabolism in the detoxification of EDP by effects of its lipophilic character was confirmed, but no importance was given to the US-assisted step that was developed following existing literature [71].

4.2. The effect of US on no hydrolytic enzyme-catalyzed reactions

Most of the slow biocatalyzed reactions that have been intended to be accelerated by US energy during more than 20 years have involved lipids, the products from which are applied on different fields (*e.g.,* foods, cosmetics, pharmaceuticals, biofuel). The variety of the substrates and products in each field is so wide that general guides on the behavior of them when subjected to US energy have yet not been established. Some examples of progresses in some of these fields are discussed below.

4.2.1. Enzymatic esterification assisted by US

Esterifications constitute one of the most representative group of enzymatic-assisted reactions the slowness of which has promoted the study of US application for acceleration. Interesterification of *N*-acetyl-phenylalanine ethyl ester catalyzed by subtilisin was developed for the first time in 1991, and substantial increase of the enzyme activity by US pretreatment was demonstrated [6]. The effect seemed to be dependent on US amplitude, the presence of water in the reaction medium and length of the chain of the alcohol involved. Subsequent studies on acylations and transacylations attributed the accelerating effect of US to the increase in temperature this energy causes, which also degrades the biocatalyst over a certain value. The increased pressure and active surface of the enzyme were also considered influential variables [16]. After more than 20 years, during which the research in this field has increased almost exponentially from 1-to-2 publicationes/year before 2004 to more than 25 in 2014, the results obtained cannot allow general application of given working conditions. Therefore, each piece of research establishes the conditions for working on given systems, for given ranges of the influential variables and for given optimization designs, either uni- or multivariate approaches.

An exhaustive study on the enzymatic acylation of troxerutin by alkaline protease from *Bacillus subtilis* with the assistance of US showed that, after optimization of several factors such as US application using different powers and frequencies (100, 150 and 200 W, and 40, 80 and 100 kHz, respectively), US pretreatment of the enzyme for 8 h yielded conversion of troxerutin of 87.3% as compared with 56.3% of the stirring method. The acylation position in the series of mono-substituted products thus obtained was established by ^{1}H NMR and ^{13}C NMR, but no conclusions on the effects of US variables on the nature of the reaction products were obtained [41].

Acidolysis (a two-step reaction in which TAGs are first hydrolyzed into diacylglycerols and monoacylglycerols, and then the new fatty acids are esterified into the glycerol skeleton to form new TAGs [72]) is a general enzymatic methodology more commonly employed than interesterification for the production of structured lipids, particularly of 1,3-dioleoyl-2-palmitoylglycerol (OPO), as their products are easily separated from the final reaction mixtures, thus decreasing separation costs [73]. The study recently carried out on three commercial lipases immobilized on different materials and with US assistance either during catalysis or as pretreatment step showed that under the optimum working conditions (US pretreatment for 6 min at 20 kHz, 115 W and 3 s/9 s duty cycle, 1:8 tripalmitin–oleic acid molar ratio, 12% enzyme dosage and 50 ºC temperature) the OPO yield was 51%. Fig. 2 shows the different behavior of the immobilized lipases (Fig. 2A) and the increase of OPO with time both helped by conventional stirring and under US pretreatment (Fig. 2B). Reusability of the biocatalyst up to 10 times makes the process potentially adaptable to industrial scale after checking other US frequencies different from 20 kHz [56].

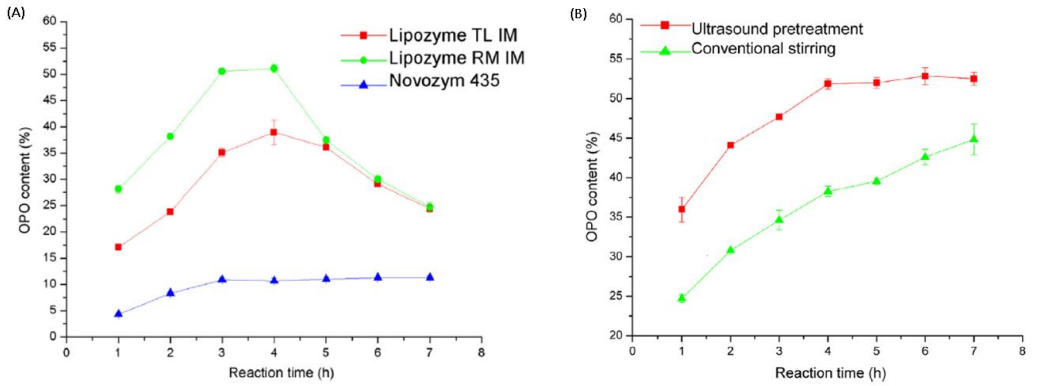

Fig. 2. (A) Effect of reaction time on OPO content using different lipases. Reaction conditions: 50% US power, 6 s/3 s ultrasonic pulse mode, 6 min pretreatment time, 1:6 tripalmitin/oleic acid molar ratio, 8% enzyme dosage and 3 mL of hexane with molecular sieves 4A (50 mg/mL), 55 ºC. **(B)** Comparison between US pretreatment and conventional stirring method on the production of OPO. US reaction conditions: 50% US power, 3 s/9 s US pulse mode, 6 min pretreatment time, 1:8 tripalmitin/oleic acid molar ratio, 12% enzyme dosage and 3 mL of hexane with molecular sieves 4A (50 mg/mL), 50 ºC. (With permission of Elsevier, Ref. 56).

Transesterification for production of 1-glyceryl benzoate from methyl benzoate under US application using a commercial immobilized lipase, Novozym 435, was optimized by a multivariate design (a full central composite rotable design) and the highest conversion, around 16%, was obtained at 65 ºC, 1:1 of methyl benzoate to glycerol molar ratio, 15 wt% of enzyme concentration, 3 mL of solvent and 40% ultrasonic power in 4 h of reaction [32]; nevertheless, the unique operating US frequency was 37 kHz and the maximum US power was 132 W, which could be not enough to obtain the attainable conversion by either higher or lower frequencies, under which higher conversions could be obtained. Similarly, the transesterification reaction for production of isoamyl butyrate from isoamyl alcohol and butyric acid using immobilized Novozym 435 and US assistance provided maximum conversion (96%) at 25 kHz frequency and 70 W US power, with shortening of the time required to 3 h *vs* 10 h in the absence of US [74].

Nevertheless, only two US frequencies (25 and 40 kHz) were studied and other variables such as US power, US application time, system temperature, etc., all them inter-related, were studied by a univariate method. Therefore, the optimum values thus obtained (including reusability of the immobilized enzyme for 7 times) could be very different by applying a multivariate approach for optimization and a wider range of US frequencies. What it is clear in any case is that US favors the reactants–enzyme contact in solvent free systems that contribute to flavor production in a greener way.

A recent example of research on transesterification is that of glycerol to glycerol carbonate (GlyC) from dimethyl carbonate also using commercial immobilized lipase (Novozym 435) and a US water bath using 20 and 40 kHz and 200 W. Evaluation of the effects of temperature, molar ratios of substrates, enzyme loading, duty cycle and US power on the conversion of glycerol to GlyC provided optimum conditions under which a 99.75% conversion and reduction of the reaction time from 14 to 10 h were achieved, with decay in both enzyme activity and product conversion with repeated use of the biocatalyst [75].

Some other organic reactions involving esterification aimed at obtaining a given product are more considered as synthesis of the pursued product than as the given organic reaction. Such is the case with the synthesis of flavonoid esters from unsaturated fatty acids to favor solubility of flavonoids such as rutin and naringin into lipophilic media that was catalyzed by Novozym 435 under US pretreatment. An ultrasonic frequency of 25 kHz, power of 200 W for rutin and 150 W for naringin and time of 1 h was determined to guarantee satisfactory degree of esterification and lipase activity. Under the optimum

conditions, the overall esterification reaction time using the ultrasonic pretreatment step was 24 h shorter than that of mechanical stirring process (48–72 h), without damaging the lipase activity [55]. Similarly, the US-accelerated enzymatic synthesis of caffeic acid phenethyl ester (CAPE) from caffeic acid and phenethyl alcohol was investigated using commercial immobilized Novozym 435. The results indicated that the reaction time, substrate molar ratio, and ultrasonic power significantly affected percent molar conversion, whereas enzyme amount did not. A model for synthesis of CAPE was established based on ridge max analysis, the optimum condition for CAPE synthesis was predicted to be reaction time 9.6 h, substrate molar ratio 1:71, a 40 kHz frequency and US power of 2 W, yielding a molar conversion of 96.03±5.18%. Experiments performed under these optimal conditions yielded a molar conversion of 93.08±0.42% [76].

4.2.2. US-assisted catalyzed biodiesel production

Despite the reactions involved in biodiesel (BD) production are common transesterification reactions, the restrictive requirements of the parameters to meet international standards for BD quality [77], and the achievements in this field by using US energy and enzymes make convenient a separate discussion of the subject. Chemical acid- and base-catalyzed, and enzyme-catalyzed BD production has been widely reported [78]. Acceleration of the former by US has been [79–82] and is at present [83,84] a matter of research. More recent is the contribution of US-assistance to biocatalyzed BD production that foreseeably will be place to abundant and fruitful research. After the first attempts by Yu *et al.* and Kumar *et al.* [47,80], the research by Feiten *et al.* has not reported useful information in this field. Following its previous experience, the group optimized typical parameters such as US power, temperature and type and reusability of the enzyme working at a single US frequency of 37 kHz and on only soybean oil as fat raw material. The group worked both in batch [54] and fed-batch regimes [85], but the produced BD did not reach the required international standard yield as the highest value they found was ~90% w/w *vs* the minimum allowed of 96.5%. The use of ethanol instead of methanol gave place to fatty acid ethyl esters considered at present an option better than methanol for making BD fully produced from renewable materials. Transesterification of waste cooking oil by dimethyl carbonate in solvent free system and in the presence of immobilized enzyme Novozyme 435 was developed under three working conditions: with only stirring and under the action of US without and with stirring. Despite the last conditions provided the highest conversion

into fatty acid methyl esters (FAMEs) (86.61%) it was far from the minimum required by international standards [86]. A higher BD yield (96%) was achieved by the same authors after US application to the same immobilized biocatalyst (no specified support) but different raw materials (Nagchampa oil and methanol) in the presence of water, different US frequency, power and duty cycle; therefore, comparison of the results was impossible [87]. Titration was the only technique used for analysis in this case (*viz.,* acid-base titration to know the amount of butyric acid produced by lipase hydrolysis, the amount of free fatty acids in the oil and the unreacted KOH); while HPLC–molecular absorption detection [86] and GC–FID [54] [85] were used in other cases; therefore, information on the reaction products was never obtained. Measurements of a crucial parameter as US power received by the sample under the experimental conditions were not performed in any of the studies [84].

5. Polymers as substrates of US-assisted enzymatic catalysis

Polymers have been subjected to the combined application of US and enzymes mainly to facilitate solid–liquid extraction (more properly named as leaching or lixiviation) of metabolites, desirable or undesirable compounds, fractionation of the polymer into smaller polymers or monomers, etc. Some key examples of the process (named as US-assisted enzymatic extraction or USAEE) are described below and others are summarized only discussing some outstanding aspects.

A method for enzymatic extraction of high quantities of different fractions of polysaccharides from given vegetables requires the use of enzymes according to the types of target polysaccharides to be fractionated. In the case of polysaccharides in leaves in a Chinese medicinal plant, papain, pectase cellulose and α-amylase were required. The enzymes together with the use of US to favor their action were named by the authors as “ultrasound complex enzymes” or “UCE”, while the extracts were considered as “the extraction solutions”, obtained from a solid leaves-extractant system considered as “a mixture”, “extracted in an ultrasonic cleaner, using selected extraction temperature ultrasound time, ultrasound power and pH” (this last sentence containing all information about the characteristics of the US device used) and using a Box–Behnken design and statistical analysis for optimization. Then, the authors applied a number of analytical methods to obtain information about the nature of the obtained extracts: total

carbohydrate content, total protein and total uronic acid by photometric methods; composition in monosaccharides by FID detection after derivatization and individual separation by GC, the average molecular weight of the fractions by high-performance size-exclusion chromatography with refractive index detector and antioxidant activity assays (DPPH radical scavenging, superoxide radical scavenging, ferric-reducing antioxidant power), immunomodulatory activity assays (on animals and cells, *in vitro* lymphocyte proliferation and macrophage phagocytic, and natural killer cell activity). The authors justified the presence of each extracted fraction by the action of one or more of the enzymes in the UCE, and attributed the favorable effect of US on the characteristics of the extracts to the cavitation phenomenon (thus, the frequency of the US transducer should be within the power zone). In short, the research is a clear example of the necessity of interdisciplinarity to obtain really useful, well supported results from a wide study, and with the correct use of the terms [88].

Biocatalysis from cellulase, hemicellulase and pectinase are widely used in juice and wine processing to degrade cell walls and plant tissues for improvement juice yield [89] and shortening the maceration time while increasing the extraction yield [90]. The extraction yield is improved by combining US and enzymes, as in the case of grape and acerola juice [91,92]. A recent contribution in this area is the research on the effect of US frequency, enzyme concentration and maceration time as variables on the USAEE of mulberry must by using the response surface methodology. Both a tunable US probe and a US bath were used to ultrasonicate the samples at various times and frequencies according to the experimental design, at constant power of 60 W with duty cycle of 10 s/15 s. Total phenols, flavonoids and anthocyanins contents were monitored together with chromatic properties of the extracts, without other information about the extracted compounds and the potential degradation caused by US. Thus, the application of a Box–Behnken design and statistical analysis special software package does not allow achieving conclusions about the quality of the ultrasonicated extract as compared with a no ultrasonicated extract, of crucial importance in dealing with food [93].

Degumming is also the name given to an extraction process that modified the surface of natural fibers such as silk by removing an amorphous matter as sericin that acts as gum binder from the main single protein known as fibroin, thus eliminating the harsh and stiff touch of the raw silk that hides the rich luster and whiteness of the commercial fiber. Traditional chemical degumming methods are highly contaminants, while the use of

enzymes overcomes this problem and the assistance of US to biocatalysis accelerates the process. A study on this matter developed in 2010 shows contradictory scientific rigor as the authors used for US application a cleaning US bath with an unknown frequency (which was not in the article or in the US cleaner catalog), but then, the effect of the US energy on the characteristics of the treated fiber was analyzed by SEM. Therefore, the exhaustive studies for comparison of the degumming process assisted by a surfactant, by US, by US+surfactant, and US+enzyme can be hardly reproduced; nevertheless, the results obtained by the US–enzyme binomial are promising as it seems to be effective and the joint action of US and the biocatalyst does not affect fribroin structure [21].

Bioethanol production from lignocellulosic biomass makes mandatory pretreatment to break up lignin structures and enhance enzymatic saccharification of cellulose. With sugarcane tops as raw material, US and a surfactant were used in the pretreatment for effective removal of hemicelluloses and lignin and improvement of the reducing sugar yield. Despite no information is given on the US device, the values of power and duty cycle were selected before applying an optimization design involving surfactant concentration, sonication time, biomass loading and incubation time as variables. The high yield of subsequent enzymatic hydrolysis obtained (0.661 g of reducing sugar/g of pretreated biomass) deserved a detailed study of the structural changes by comparing the native and pretreated biomass by SEM, XRD and FT-IR. The results of these analyses showed that, similarly to the results of previous studies, removal of some external fibers by destruction of the cellulose–hemicellulose lignin network generated a pretreated biomass more readily hydrolyzed, but the ultrasonication conditions did not hydrolyze the biomass [94]. As usual, the lack of information about the source and frequency of US hinder proper use of the results from this research.

Studies in which US has been applied as sample preparation for subsequent biocatalysis have shown that the modification of the substrate structure facilitates enzymatic action. As examples, US-assisted pretreatments of lignocellulosic biomass from wood wastes [95], sugarcane bagasse [96,97] and kenaf powder [98] undergo a milling process of the lignocellulosic substrate due to shock waves generated by the cavitation collapse that increase the surface area thereby improving enzymatic saccharification. Study of the structural changes promoted by US carried out by SEM, X-ray diffraction (XRD) and FT-IR have been developed under not well specified US conditions (“40% amplitude, pulse 59 s on and 59 s off”, without information of US frequency, power and intensity, and

involving an erroneous concept of pulse, that is the name the authors gave to duty cycle) and in the presence of surfactants. The pretreatment efficiency was measured through the reducing sugar yield after enzymatic saccharification of the pretreated sample. Scanning electron micrographs showed that US pretreatment induced physical changes in the biomass with a highly distorted structure and removal of some of the external fibers by destroying the cellulose–hemicelluloses lignin network. US does not hydrolyze the biomass to soluble sugars, but generate a pretreated biomass that is more readily hydrolyzed by increasing accessible surface area [94]. Identical observation was earlier reported for rice straw pretreated with an organosolvent [99]. Carbonyl bands also indicated removal of hemicelluloses and the X-ray diffraction profile showed an increase of the crystallinity index, which is also enhanced when surfactants are present in the media, and caused by an effective removal of amorphous components like lignin and hemicelluloses. A key aspect of US application prior to introduction of enzymes into the medium under study is that changes in the structural features of pretreated *vs* native samples facilitate subsequent enzyme biocatalysis and avoid the degrading effect this energy can exert on the given enzyme.

Research on evaluation of pretreatment of sugarcane bagasse combining US and supercritical carbon dioxide (SC-CO_2) in a sequential manner to enhance the enzymatic hydrolysis of pretreated bagasse has been developed by Benazzi *et al*. After multivariate optimization of the SC-CO_2 pretreatment, the authors improved the amount of fermentable sugar of about 280% as compared with no treated bagasse, conducting to hydrolysis efficiencies of 74.2%. When this pretreatment was combined with previous US application the amount of fermentable sugar increased 16%. The authors supported the improvement provided by SC-CO_2 on the agreement with previous results and on US cavitation [100].

Ring-opening polymerization of lactones and lactides had previously been achieved by chemical- and bio-catalysis helped by energies such as microwaves [101] or by supercritical fluids [102] in the presence of ILs [103]. The use of US in combination with enzymes has been applied to the *Candida antarctica* lipase B mediated ring opening polymerization of ε-caprolactone to poly-6-hydroxyhexanoate, also with the assistance of an IL (1-ethyl-3-methylimidazolium tetraflouroborate). The authors compared the method with and without US assistance and checked that in the former case monomer conversion increased by 63% and afforded a polymer product of a narrower molecular weight distribution and a higher degree of crystallinity. Under US application, the polydispersity

index of the product was ~1.44 compared to a value of ~2.55 for the product from the conventional reaction. The improvement was associated to US microstreaming that favored mass transfer of the monomer to the active site of the enzyme in a reaction mixture that became increasingly viscous with time and, therefore, increasingly limited mass transfer [104]. Despite data about the US device used (35 kHz and 320 W) appears in the article, no information about optimization nor values of the applied US variables are given. The authors analyzed the treated samples by FT-IR, ^{1}H NMR, gel permeation chromatography, differential scanning calorimetry and thermogravimetric analysis, and evaluated the volumetric productivity and monomer conversion, but they did not compare the results they obtained with those provided by assistance from other energies [105].

One of the scant contributions in polymers treatment of US multifrequency devices is that developed by Oliver *et al.* for biodegradation of wheat chaff. The authors used a sequential two-steps US-assisted pretreatment of the sample (10 min each) at 40 and 270 kHz, but they did not justify the selected frequencies, the US application time or the necessity for two sequential treatments to increase enzyme accessibility to the cellulose fibrils by disruption of the plant surface structure, as shown by changes in the microestructure. Then, they applied US (80 kHz) to the extract–enzyme system for 72 h with a duty cycle of 1 min/31 min and analyzed the resulting analytical sample by head space GC–MS to obtain the mass profile of volatiles. The scheme of the overall process includes a previous treatment with microwaves, which was not useful at all [106].

Studies on US treatment of sorghum slurry prior to liquefaction and saccharification under optimized conditions provided an increased yield of glucose within 10–25%, depending on time of US application and intensity (horn working at 20 kHz and 750 W maximum power). The reason for the increase in the percentage of saccharification was attributed to the availability of additional starch for hydrolysis due to US-assisted disruption of the protein matrix (surrounding starch granules) and the amylose–lipid complex [29].

US-assisted enzymatic extraction of arabinoxylan, the major dietary fiber component of wheat bran, has recently been reported [107]. Commercial enzymes (endo-1,4-β-xylanase, α-amylase, protease, and amyloglucosidase) acted on the sample in a sequential manner under the action of US provided by a US bath equipped with two US transducers with frequencies 28/40 and 50/135 kHz. Despite the possibilities to study the influence of the different US frequencies on the different enzymes, the authors only applied

and validated an optimization design and presented the tables of data, three- and bidimensional plots with no mention to the effect of US on both solid raw material and enzymes. The analytical equipment involved in the research was quite similar to that used by Wu *et al.*, who obtained the analytical information by a common photometer; therefore, information only concerned to overall common parameters and no to the effect of US assistance as a function of the values of its characteristic parameters [108]. It is clear that despite a multivariate design is useful for optimization, information on the effect caused by the auxiliary energy is mandatory to evaluate its usefulness.

A recent comparison of the influence of US on the chemical and enzymatic hydrolysis of yam has been reported. The comparison was only based on the yield of fermentable sugars: the highest yield was obtained by the US-enzymatic process, followed by that enzymatic, then US–chemical and chemical [109]. This would be an excellent system to study the pathways followed by hydrolysis in each case for which the variables involved in the optimization designs provided scant or null information.

6. Proteins as substrates of US-assisted enzymatic catalysis

The subject of this section was widely covered by Bermejo *et al.* in 2004 in a review on enzymatic digestion and ultrasonication [110]; therefore, only some specific, recent contributions are discussed below.

The analysis of protein-containing samples requires a pretreatment for deproteination, which is usually accomplished by acid treatment (trichloroacetic acid, TCA), organic solvents (methanol, acetonitrile) or heat. However, these treatments seem to be not very efficient to remove this interference, particularly when the analytical method uses a mass detector for identification and/or quantitation [111]. To overcome this problem, a US-assisted enzymatic hydrolysis method was developed to break the three dimensional structures of proteins and release bound tetracycline antibiotics (TCs). This step not only improved significantly the recovery of TCs, but also shortened the pretreatment time from 16 h to 6 min. By combining this new deproteination method and a solid phase extraction clean-up step, a suitable sample preparation was achieved for the analysis of TCs by LC–MS/MS. The overall method provided recoveries in the range 89.1–102.4%, much higher than those obtained when acid deproteination was used (23.5–36.2%). Despite the "ultrasound-assisted enzymatic hydrolysis" of the proteins was even in the title, no

information is given about the US device used nor about its frequency, but only about the optimum US power (100 W), above which the efficiency of the process decreased. The drastic reduction of the time required for protein enzymatic digestion in the presence of US as compared with that necessary in the absence of this energy deserves an in depth study of US action, which clearly was not the aim of this research [23].

Enzymatic hydrolysis by pepsin of seafood materials to liberate arsenic species (arsenite, As(III); arsenate, As(V); dimethylarsinic acid, DMA; and arsenobetaine, AsB) As(III), As(V), DMA and AsB) was performed with US assistance by a US water bath working at 35 kHz, thus shortening the digestion time from 12–14 h to 4–30 min. The digested thus obtained, after being subjected to a clean-up step, was injected into an LC for individual separation of the target analytes prior to atomization–detection by ICP–Orbitrap MS [10]. The authors used microwave-assisted digestion (MAD) as the standard method for comparison with the proposed method, and they obtained good results. Nevertheless, the authors did not justify why they used the US frequency of 35 kHz and not that at 17 kHz, also provided by the US water-bath; and why the times required for hydrolysis of the different arsenic species were also different. Similar studies have later been developed by the same authors for determination of iodinated amino acids in edible seaweed using in this case pancreatin as biocatalyst. Also similar was the comparison of the proposed US-assisted method with the MAD method as standard, but the US bath worked at 45 kHz as single frequency in this case. No explanation nor discussion was given about the difference in percent content of iodine between the two target iodinated species (50–83% of the extractable iodine) and the total content that the authors attributed to no extractable iodinated species and/or minor un-extractable target amino acids [112].

A more complete study on the effects of US treatment during proteolysis on kinetic characterization of the hydrolysis of defatted wheat germ protein (DWGP), and on the inhibitory activity of the hydrolyzate of the angiotensin I-converting enzyme (ACE) has been developed [113]. Thus, the effects of US pretreatment on the release of peptides with ACE-inhibitory activity showed that the value of k_A for DWGP hydrolysis under US application increased by about 22.2%, and K_M decreased about 13.0%, compared with that obtained without US. The analysis of ACE-inhibitory activity indicated that US had less effect on the ACE-inhibitory activity during enzyme treatments than US pretreatment that caused a 21.0–40.7% increase in ACE-inhibitory activity of DWGP hydrolyzate. The studies on hydrophobicity, microstructure, and amino acid composition revealed that US

pretreatment could result in an increase of surface hydrophobicity of DWGP and loosening of protein tissue, which accelerate the release of hydrophobic amino acids during enzymatic hydrolysis, thus facilitating the enzymatic hydrolysis of DWGP; whereas US pretreatment could promote the release of ACE-inhibitory peptides from DWGP during enzymatic hydrolysis. The digested was subjected to RP-HPLC separation of the target amino acids previously derivatized for final fluorescence detection. A single frequency (20 kHz) was provided by the US probe working at a duty cycle of 2 s/4 s, but the applied power was provided as power output rather than fractions of the maximum power provided by the probe. Also, US pretreatment was the best option for subsequent treatment of peanut kernels with trypsin and α-chymotrypsin to increase the solubility of peanut protein and decrease the concentrations of allergenic proteins. No explanation was provided on the phenomena caused in the raw material by US pretreatment [114].

Studies on garlic have been carried out mainly by Chinese researchers, possibly as a consequence of being China the main producer of this edible plant. Studies by Peng *et al.* [115] showed that garlic powder treated by US and alcalase increased the ACE-inhibitory activity by 170.9% over the water extracts. This behavior led Huang *et al.* to evaluate the effects of US pretreatment on kinetic constants of garlic protein hydrolysis by alcalase [116]. The aims were to study the effects of optimal ultrasonic conditions on the kinetic constants of garlic enzymolysis, and determine the hydrolysis curves of garlic catalyzed by alcalase in solution, modeled by a simple empirical equation from which kinetic parameters can be deduced. The final aim of the Huang's group was to provide the theoretical basis and technological support for better understanding and designing enzymatic hydrolysis of other ultrasonic pretreated materials. Nevertheless, they only worked at a fixed frequency of 20 kHz, US power of 410 W duty cycle of 3 s/5 s and 8.2 initial pH in a countercurrent system, and determined the kinetic parameters from the data provided by a colorimetric method for determination of total phenols. With these data, and those from the traditional enzymolysis method, the kinetic parameters were calculated and compared. They confirmed the accelerating effects on the enzymatic action of US pretreatment of the substrate; in this way, the enzyme was not modified by this energy.

7. Effects of US on enzymatic degradation

Studies on acceleration of enzymatic catalysis by US have been mainly aimed at

obtaining a given product in a shorter time, as is the case with metabolites, amino acids or peptides, as the most outstanding examples. Nevertheless, research on the US-enzyme binomial for degradation of undesirable compounds or materials also deserves discussion.

Wastewater discharge from textile industries contains many types of dyes that are toxic organic pollutants. Low-frequency US has been used to degrade undesirable contaminants by effect of the cavitation phenomenon and the formation of free radicals. Also peroxidases have been used to catalyze the oxidation of a variety of contaminants using H_2O_2 as an electron acceptor. The combined action of US of 20 kHz frequency, peroxidases and high static pressure on dyes degradation has been studied with the following main conclusions: the interaction between US waves and enzyme is of physical nature; intense convection generated in the bulk liquid medium enhances the interactions between dyes and enzyme molecules and yields enhanced degradation; the oxidation power of horseradish peroxidase to degrade dyes is a function of the convection level in the medium; the highest synergism between US and enzymatic degradation of the dyes organic pollutants is achieved in the absence of cavitation phenomena, as US provides intense convection in the medium, and increases the probability and energy of interaction between the dyes and the enzyme [117]. Cavitation by itself contributes to dyes degradation through the OH radicals produced during transient collapse, but this effect works against the enzyme action by denaturation. High convection level in the medium obviates the need for external agents such as polyethylene glycol that would prevent deactivation of enzyme.

The contaminant leather industry can also take advantage of the US–enzyme binomial for degradation in two preliminary steps of the whole process: acting on the untanned leather waste [30] or by converting the unhairing step into a clean step [26]. In both cases, US favors penetration of the enzyme through the skin. In the first case, the cementing substances known as “proteoglycans” or as “mucoids”, mainly composed of protein and polysaccharides, are degraded, and protein and saccharides are liberated into the enzyme bath [118] by the action of amylase and protease enzymes and that of US of frequency and intensity of 20 kHz and 2.2 W/cm^2, respectively, with little effect on both the enzyme activities and on the physical and mechanical properties of the leather. No difference was detected on the use of two US frequencies, 20 and 40 kHz. On the other hand, fleshings and shavings from the tannery industry when treated with alkaline protease (less sensitive to US effects than neutral protease) and US of 40 kHz and 0.64 W/cm^2

yielded a conversion ratio of the enzymatic hydrolysis of 84.1% as compared with the 57.6% obtained in the absence of US. The reason of this increased yield was ascribed to the dual effect of US by facilitating passage of the protease through the skin pores and promoting the breakdown of helical regions of collagen, which are difficult to attack by proteases.

An example of the potential of the degradation effects of combined US and enzymes to minimize contamination of aquatic ecosystems by pharmaceuticals is the recently reported method for degradation of cetirizine dihydrochloride, an antihistaminic drug. Using laccase and US (after optimization of parameters such as enzyme loading, temperature, US power and frequency, duty cycle, and speed of agitation) a maximum degradation of 91% was achieved in 7 h, while with the conventional stirring method the degradation was about 13% in 24 h. The pathway proposed for degradation of the target drug was confirmed by MS [25].

8. Inhibition/activation of enzyme effects by US

Inhibition or activation of enzyme catalysis by US can be either a pursued effect or an undesirable effect.

Enzyme inactivation is a requisite for stabilization of some materials, particularly foods. Although inactivation can be easily achieved by heat treatment, there are a number of cases where the high heat resistance of some enzymes makes heat treatments not a solution of the problem but a problem by itself, because heat can negatively modify some food properties such as flavor, color or nutritional value. The efficiencies of nano-thermosonication (MTS) —a combination of heat, moderate pressure and ultrasound (50–130 ºC, 0.2–0.3 MPa, 117 μm amplitude)— against the activity of two proteolytic enzymes and two lipolytic enzymes (porcine pancreatic lipase and phospholipase A, and α-chymotrypsin and trypsin), which differ considerably in their heat resistance although they catalyze similar reactions, was studied, and very different results were obtained depending on the biocatalyst. Thus, whereas phospholipase A2 was almost insensitive to MTS treatments, α-chymotrypsin and porcine lipase MTS inactivation was much faster than heat inactivation. MTS is also able to impair the protection that several molecules offer to several enzymes against heat inactivation. As a result, MTS is also able to inactivate enzymes that are protected by such molecules at high temperatures. Molecular size and structure play also a role in the sensitivity to MTS; large and less globular enzymes being more sensitive

to MTS. Sensitivity to MTS is the result of a complex interaction of heat resistance, molecular structure and size, and treatment medium. Titrimetric, photometric and fluorimetric monitoring of the effects did not provide the required information for appropriate discussion about the mechanisms through which inactivation occurred in each case. No experiments in the absence of applied pressure and heat were developed [7]. Heat and MTS treatments were carried out in a TR-SC thermoresistometer [45].

A recent study has also combined US and pressure —high hydrostatic pressure (HHP)— on enzymes (polyphenolase, peroxidase and pectin methylesterase) for the production of safe and quality fruit juices in which phenols, flavonoids and flavonols should remain unaltered. Unfortunately, the analytical assays performed (determination of the residual activity of the enzymes after US–HHP treatment, determination of ascorbic acid content, total phenolics, flavonoids and flavonols, total antioxidant capacity and DPPH radical scavenging activity, color, pH, soluble solids and titratable acidity) were all unspecific assays that did not provide information on the products resulting from the application of US–HHP. The overall, indiscriminate information could hide degradation of the components of interest in the fruit juices, as some values of overall parameters even increased in the products as compared with that of the original compounds [119].

A research providing contradictory results that require a more in depth study with appropriate analytical instrumentation was the evaluation of the effects of US on the activity of α-amylase and amyloglucosidase by the results of a multivariate optimization design involving temperature and pH in the presence and absence of US, as they behaved differently to alterations of both variables. For temperatures up to 50 ºC the enzymes activities were always higher in the presence than in the absence of US, but above 60 ºC the activity in the absence of US was always higher. The activation energy of both enzymes in the presence of US was decreased considerably. The study of the influence of changes of physical and chemical variables on the structure of the enzymes (and thus on their inhibition or activation) was only evidenced, but not confirmed by using appropriate analytical equipment to support the experimental findings [53].

Because the growing use of fresh-cut vegetables, investigation about the combined effect on activity changes of polyphenol oxidases and peroxidase (as enzymes involved in the browning process) with US and ascorbic acid on fresh-cut apple during storage has been developed. The combined treatment inactivated monophenolase, diphenolase and peroxidase, whilst the individual treatment with US or ascorbic acid had inverse and

limited inhibitory effect on the enzymes. Photometric monitoring and conventional gel electrophoresis are not the most appropriate analytical tools to elucidate the changes the individual and combined application of energy and chemicals produced on the biocatalysts. Information about the type of US device used (only the frequency) does not help to a correct interpretation of the results [120].

Information about changes produced by US (40 kHz, 300 W at temperature in the range 0–4 ºC) on structures of α-amylase and other proteases as papain and pepsin has recently been reported. The effect of US on the activity of the enzymes has been investigated and the mechanisms involved have been explored. Conformational changes in enzymes structures, folding and binding properties were determined by CD; FT-IR was used for the estimation of their three-dimensional structures and fluorescence measurements as an effective indicator of tertiary structure transitions in proteins. It is worth clarifying that the fluorescence behavior of a protein is mainly attributed to its aromatic amino acid residues, especially the tryptophan residue; therefore, tryptophan fluorescence is considered reliable to determine the conformational changes of proteins caused by US [121]. Fig. 3 confirms that inhibition of enzyme activity by US was highly related to enzyme structure: after 60 min of US application the activity of native amylase decreased from 627.76 to 107.61 U/mg (Fig. 3A); that of papain was reduced from 21.33 to 15.05 U/mg, while the activity of pepsin significantly increased from 46.46 to 53.22 U/mg (Fig. 3B). The activity change of these three enzymes seems to be mainly due to the variation of their secondary and tertiary structures.

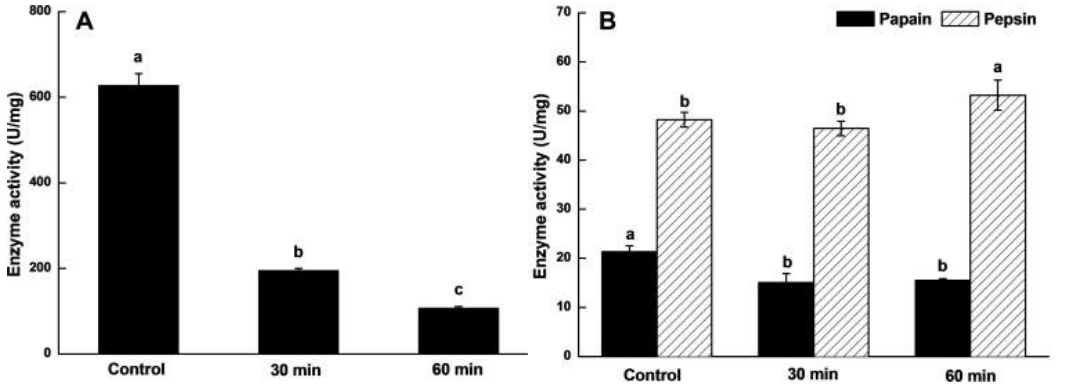

Fig. 3. Effect of US application on, **(A)** α-amylase activity, **(B)** papain and pepsin activity. (With permission of Elsevier, Ref. 121).

In short, low-frequency US mainly promotes inhibition of enzymatic activity, probably caused by cavitation and shear forces that should be investigated by proper analytical tools to provide information on the conformational changes produced in the biocatalysts as a function of their structure. Additionally, higher US frequencies should be applied to prove their influence that could be very different.

9. US-assisted enzyme production and related processes

US is a useful tool to assist different steps involved in enzyme production. Thus, enzyme production, extraction, purification, and immobilization can be improved by US application.

Conventional production of fibrinolytic enzymes in liquid–in suspension particle systems is favored by aeration and agitation that decreased oxygen depletion and increase mass transfer, respectively. Phenomena associated to low-frequency application were considered to provide a higher efficiency by favoring the production process. With this aim, a single US frequency of 25 kHz was used, while other variables such as US power and duty cycle, and application time were optimized using a multivariate approach [122]. Photometric monitoring of both the decline in glucose concentration and protease activity did not provide information on how US acts to promote an 1.48-fold increase in fibrinolytic enzyme yield as compared to no ultrasonicated fermentation. Therefore, more resolutive analytical equipment would be necessary to study the modification produced by US energy, which should be complemented by using different US frequencies for a better understanding of how US can better help to production of this enzyme.

A more in depth study was also aimed at enhancing the production of a fibrinolytic enzyme by US stimulation. Parameters such as US application at different growth phases, US power and duty cycle, US duration, and multiple US application were studied to enhance the yield of the fibrinolytic enzyme by a univariate design despite variables inter-relation. Under the optimum US conditions (25 kHz, 160 W, 20% duty cycle) applied for 5 min the productivity of the target enzyme increased by 1.82-fold (from 110 to 201 U/mL) as compared with the no ultrasonicated control fermentation. Drop in glucose concentration from 0.41% to 0.12% w/v in ultrasonicated batches implies that US increases the cell permeability, improves substrate intake and progresses metabolism of microbial cell. SEM images before and after US stimulation clearly showed the impact of duty cycle

on decreasing biomass concentration. However, environmental scanning electron micrographs does not show any cell lysis at optimum US application [123].

While solid-state fermentation process for ligninolytic and hydrolytic enzymes production can hardly be assisted by US, this energy can be used to improve the efficiency of the subsequent step: enzyme extraction. With this aim, studies about the action of low-frequency US (horn working at 20 kHz and 500 W) on the free enzymes (endoxylanase, 1,4-β-D-glucosidase, 1,4-β-D-endoglucanase, polygalacturonase, lignin peroxidase, manganese peroxidase and laccase) were developed and no significant changes were detected. Then, the enzyme activities in the extracts obtained by conventional stirring and by US at different powers were compared and increases within 129–498% were achieved under ultrasonication as a function of the increased US power. Despite the simple analytical instrumentation used, the previous experiments on free enzymes confirmed their no degradation by US; therefore, the increased activity must be ascribed to more efficient extraction in the presence of US [124].

Comparison of the influence of US frequencies on cell disruption to extract enzymes (protease, α-amylase, α-glucosidase, and alkaline and acid phosphatases) from sludge flocs showed that working at 20 kHz more amount and types of enzymes could be extracted than at 40 kHz or by the EDTA method. In addition, US intensity seemed to be more influential than duration of US application. Under the optimum extraction conditions, US could break the cells and extract both the extracellular and a small part of intercellular enzymes from sludge flocs. On the other hand, the Pearson correlation analysis between enzyme activities and cation contents revealed that the different types of enzymes had distinct cations binding characteristics [44].

Purification of fibrinolytic enzyme extracts by three phase partitioning (TPP), which integrates concentration and purification, can also be assisted by US, giving place to UATPP. A three-phase system was formed by simultaneous addition of ammonium sulfate and *t*-butanol to crude broth. UATPP of a fibrinolytic enzyme was studied by varying different process parameters such as ammonium sulfate saturation concentration, pH, broth-to-*t*-butanol ratio, temperature, and US frequency power and duty cycle. The optimized parameters yielded maximum purity of 16.15-fold of fibrinolytic enzyme with 65% recovery. SDS PAGE analysis of partitioned enzyme shows partial purification with a molecular weight in the range of 55–70 kDa. Enhanced mass transfer of UATPP resulted in higher purity of fibrinolytic enzyme with reduced time of operation from 1 h to 5 min as

compared to conventional TPP. No big differences were found in the results provided by the two assayed US frequencies (25 and 40 kHz), but 25 kHz was slightly better [125].

The advantages of immobilized enzymes as compared with their in solution counterparts are well-know, but what is scantly known is that US can facilitate and improve the immobilization process. These beneficial effects have been demonstrated by immobilization of dextranase onto calcium alginate beads. Furthermore, the activity of the free and immobilized enzyme prepared with and without US treatment, as a function of pH, temperature, recyclability and enzyme kinetic parameters, was compared. The best characteristics of immobilization were achieved when the immobilized dextranase was prepared under US application at 25 kHz, 40 W for 15 min, under which the loading efficiency and the immobilization yield increased by 27.21% and 18.77%, respectively, as compared with the immobilized enzymes prepared in the absence of US. On the other hand, US-assisted immobilization showed V_{max} and K_M values higher than without US; therefore, the former immobilization method improved the catalytic kinetics activity of immobilized dextranase under the reaction conditions studied; method that also shows higher optimum pH and reaction temperature, activation energy, and thermal stability, as well as a higher recyclability [126]. The good results obtained make desirable research on other types of enzymes and supports, other US conditions (*viz.,* a wide range of US frequencies, power, continuous *vs*. discontinuous US application, and, particularly, modifications on the enzyme structure and/or binding to the support that justify the favorable action of US on immobilization).

10. Fields of application of the US-enzyme binomial

Tables 1-to-4 intends to give an overview of the most outstanding fields of application of the binomial object of this review. In them, special emphasis has been given to recent contributions without forgetting salient no so recent studies. Common aspects of the four tables are the scant importance given to the US device (and the scant information the authors provide about it, as a result); the great importance given to the improvement US produces on the given process with no interest in the way US acts on the improvement; and the poor analytical instrumentation used, most times, to monitor the changes produced by US.

Table 1. Applications of the US–enzyme binomial in the food area.

Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device	Analytical technique	Comments	Ref.
Alginate	Dextranase	-Enzyme immobilization	Probe/25 kHz/100 W	HPLC–RID	US-assisted immobilized enzyme works at higher values of pH and optimal reaction temperature, lower activation energy, and higher thermal stability recyclability	[126]
Buffer	Sucrose	-Invertase -Hydrolysis	Bioreactor/815 kHz	Photometry	Measurements of US intensities and pressures	[15]
Meat	Biofilms	-Glycolytic, proteolytic -Hydrolysis	No commercial curved probe/40 kHz	Standard plate counting	Removal of *E. coli* and *S. aureus* biofilms	[46]
Buffer	Proteins	-Tryipsin -Digestion	Reactor, Probe	MALDI–TOF–MS	Influence of US device with different characteristics	[43]
Tomato	Peroxidase	-Peroxidase -Inactivation	Probe/23 kHz	Photometry	POD activity regeneration on storage	[127]
ILs	Sugars	-Lipase -Esterification	Bath	HPLC–UV, NMR	ILs favor US action	[128]
Potato, oyster	Starch, glycogen	-α-Amylase, amyloglucosidase -Hydrolysis	Bath/60 W	Photometry	Determination of optimum power density	[20]
In solution enzymes	Enzymes	-Phospholipase A2, α-chymotrypsin, trypsin and porcine pancreatic lipase -Hydrolysis, esterification	Manothermo-sonicator (non comercial)	Photometry	US+ pressure application	[7]
Soy oil	Triacylglycerides	-Lipase -Hydrolysis	Bath/28 kHz	Titration	Solvent-free reaction	[31,62]
Non-aqueous	Glucose	-Lipase -Transesterification	Reactor/20 kHz/variable power	Photometry	Unchanged enzyme selectivity under US	[22]

Continuation Table 1

Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device	Analytical technique	Comments	Ref.
Sorghum flour	Starch	-α-Amylase, amyloglucosidase -Hydrolysis	Probe/20 kHz/ 750 W	Photometry, HPTLC–no specified detector	US action: sorghum flour directly for liquefaction and saccharification	[29]
Isooctane	Caffeic acid, 2-phenyl ethanol	-Lipase -Esterification	Bath/40 kHz	HPLC–UV	Synthesis. Molar conversion of 96.03± 5.18% in 9.6 h	[76]
Pyridine	Flavonoids acylation (troxerutin)	-Alkaline protease -Transesterification	Bath/40,80,100 kHz/ 200 W	HPLC–UV	US application to protease prior to acting on synthesis. Unchanged regioselectivity.	[41]
Organic solvents	Palmitic acid, ascorbic acid	-Lipase -Esterification	37 kHz/132 W	HPLC–RI	US-assisted maximization of ascorbyl-palmitate synthesis	[129]
Olive oil	Triacylglycerols	-Lipase -Glycerolysis	37 kHz/132 W	Derivatization +GC–FID	US-assisted formation of mono- and diacylglycerols	[35]
Flaxseed	Cellulose, hemicellulose, pectin	-Cellulase, hemicellulose, pectinase -Hydrolysis	20 kHz/250 W	Photometry, titration	US-assisted extraction	[52]
Apple	Oxidase, peroxidase	-Oxidase, peroxidase -Inactivation	40 kHz	Photometry	US+ascorbic acid-assisted inactivation of fresh-cut apple enzymes	[120]
Synthetic milk salts buffers	Phospholipase A2, α-chymotrypsin, trypsin, lipase	- Phospholipase A2, α-chymotrypsin, trypsin, lipase -Inactivation	Nano-thermosonicator (MTS)	Photometry, titrimetry	MTS treatment more effective than heat	[130]
Palm oil	Triglycerides	-Lipase -Hydrolysis	Probe/23 kHz/ 200 W	Titration, NMR	US-assisted emulsification prior to enzyme addition	[18]
Potato	Starch	-Amylase -Hydrolysis	Bath/40 kHz/132 W	Photometry	US-application enhances 3-times enzyme activity, and decreases 80% the activation energy	[131]
Starch, casein	Starch, casein	-α-Amylase, papain, pepsin -Hydrolysis	Bath/40 kHz/300 W	CD, FT-IR, fluorimetry, photometry	US-inhibited activity of α-amylase and papain, and enhanced activity of pepsin	[121]

Continuation Table 1

Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device	Analytical technique	Comments	Ref.
Buffer	Tyrosinase	-Tyrosinase -Hydroxylation, oxidation	Bath/40 kHz/100 W	Photometry, FT-IR, AFM	US-assistance breaks large molecular groups facilitating substrate–enzyme contact. Avoidance of fruit browning	[19]
Organic solvent	Resveratrol, vinil acetate	-Lipase -Acetylation	Bath/40 kHz/150 W	HPLC–UV	US-assistance shortens the reaction time and increases yield	[132]
Chicken	Tetracyclines associated to proteins	-Trypsin -Hydrolysis	No data/100 W	LC–MS/MS	US-disruption of folded, native structure of proteins	[23]
Wheat bran	Arabinoxylans associated to proteins, phenolic compounds, cellulose and β-glucans of wheat bran	-Endo-1,4-β-xylanase, α-amylase, protease, alcalase, amyloglucosidase -Hydrolysis	Bath/28, 40, 50, 135 kHz/180 W	GC–FID	US increases enzymatic extraction yield of arabinoxylans from wheat bran	[107]
Crude rapeseed oil	Phospholipis	-Phospholipase A_1 -Hydrolysis	Bath/40 kHz/ 0.07 W/cm²	Titration, photometry	Shortening of reaction time, decreased water and enzyme dosage used in phospholipase degumming process	[66]
Apple juice	Phenolic compounds and pectins	-Polyphenolase, peroxidase, pectin methylesterase -Oxidation, hydrolysis, esterification	Bath/25 kHz/ 2 W/cm²	Photometry	Higher inactivation of enzymes with complete inactivation of total plate counts, yeasts and molds	[119]
Blackcurrant fruits	Crushed blackcurrant	-Peptinase, papain -Hydrolytic digestion	Probe/400 W	Photometry, GC–FID, FT-IR	US-assisted extraction of polysaccharides	[133]
Lycium barbarum fruits	Crushed Lycium barbarum	-Cellulase -Hydrolysis	Bath/40 kHz/120 W	Photometry	US-assisted extraction of polysaccharides. Agreement between the predicted and obtained yields	[134]

Continuation Table 1

Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device	Analytical technique	Comments	Ref.
Wheat chaff	MW-pretreated wheat chaff	-Rot fungi enzymes -Oxidation	Bath/40, 270 kHz	CLSM, SEM, HS–GC–FID	Pretreatment with: -Low US frequency to crack the waxy cuticle -Medium US frequency to enhance oxidation	[106]
Cucurbita moschata (pumpkin)	Solvent-free pumpkin	-Cellulase, pectase, papain -Hydrolytic digestion	Probe/20 kHz/ 900 W	Photometry	US-assisted extraction of polysaccharides with the highest antioxidant power	[108]
Garlic	Dehydrated garlic, water	-Alcalase -Hydrolysis	Reactor/20 kHz/ 410 W	Photometry	US-assisted enzymolysis of garlic proteins	[116]
Mulberry must	Mulberry must	-Alcalase -Hydrolysis	Probe, bath, transducer/ 22-42 kHz/60 W	Photometry	Shortening of processing time and improvement of must composition	[93]
Sodium carbonate solution	Freshwater clam soft body	-Papain -Hydrolysis	Bath/300 W	FT-IR, SEM and HPLC–UV	Improvement of polysaccharide yields and shortening of extraction time.	[135]
Sugarcane, surfactant	Sugarcane	-Cellulase -Hydrolysis	Bath/40% amplitude/ (59/118 s/s)	SEM, XRD, FT-IR	Surfactant+US-assisted pretreatment for hemicelluloses, lignin removal. In-depth studies on structural changes induced by US	[94]
Sugarcane	Sugarcane	-Cellulase -Hydrolysis	Bath/40 kHz/132 W	Photometry	US-assistance doubles the hydrolysis yield	[136]
Cassava	Cassava waste	-α-Amylase, amyloglucosidase -Hydrolysis	Bath/40 kHz/132 W	Photometry	US-assisted enzymatic obtainment of fermentable sugars	[137]
Organic solvent	Cellulose	-Cellulase -Hydrolysis	Reactor/40 kHz/ 500 W	Photometry	US effect on enzyme inactivation increases by increasing US amplitude	[59]

Continuation Table 1

Sample matrix	**Substrate or enzyme**	**-Enzyme -Catalyzed reaction**	**Type of US device**	**Analytical technique**	**Comments**	**Ref.**
Roasted peanuts	Proteins, aqueous buffer	-Trypsin, α-chymotrypsin -Hydrolytic digestion	Bath/50 kHz	Photometry/ ELISA	US-assisted pretreatment for allergens reduction	[114]
Seaweed	Proteins	-Cellulose, α-glucosidase, α-amylase, lipase, pepsin, pancreatin -Hydrolytic digestion	45 kHz	RP-HPLC–ICP–MS	Iodinated amino acids	[112]
ILs	Glucose	-Glucose isomerase -Isomerization	Bath/40 kHz/500 W	Photometry	Combined synergistic effect of US and ILs.	[48]
Olive oil	Triglycerides	-Lipase -Glycerolysis	Bath/37kHz/132 W	GC–FID	US-assisted production of mono- and diacylglycerols	[138]
Solvent-free palm oil	Triglycerides	-Lipases -Glycerolysis	Probe/47kHz	GC–FID	US-assisted production of diacylglycerols	[65]
Hexane	Tripalmitin, oleic acid	-Lipases -Hydrolysis, esterification	Probe/20 kHz/ 130 W	HPLC–APCI–MS/MS	Shortening of processing time, improvement of affinity between substrate and enzyme	[56]

SCF, supercritical fluid; PXRD, powder X-ray diffraction; UFA, unsaturated fatty acid; AFM, atomic force microscopy; CLSM, confocal laser scanning microscopy

Table 2. Applications of the US–enzyme binomial in the environmental area.

Target system	Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device/ features	Analytical technique	Comments	Ref.
Toxicants	Seafood	Proteins, organo-As compounds	-Pepsin -Hydrolytic digestion	Bath/ 35 kHz	HPLC–ICP/MS	Digestion shortening from 12–24 h to 5 min	[10]
Cosmetics	Urine	Sunscreen agent	-β-Glucuronidase -Hydrolysis	Probe/ 20 kHz/ 450 W	HPLC–TOF/MS, HPLC–MS/MS	Hydrolysis shortening from 12 h to 50 min	[71]
Pollutans	Organic solvents	Recalcitrant dyes	-Peroxidase -Oxidation	Bath/35 kHz/35 W	Photometry	US-assisted degradation	[117]
Pollutants	Water	Phenol	-Peroxidase -Oxidation	Bath/423 kHz/5.5 W	HPLC–UV	Phenol degradation	[37]
Pollutants	Synthetic	Phenols	-Peroxidase -Oxidation	Bath/423 kHz/5.5 W	HPLC–UV	Degradation of substituted phenols	[36, 38]
Pollutants	Wastewater	Reductant compounds	-Cellulase, endo-glucanase, cellobio-hydrolase -Hydrolysis	Bath/ 22.5 kHz	Potentiometry (pH)	Decrease chemical oxygen demand (COD)	[139]
Leather	Untanned solid leather waste	Proteins	-Alkaline and neutral proteases -Proteolysis	Bath/ 40 kHz	Photometry	Impair collagen structure	[30]
Leather	Skin	Proteins	-Neutral and alkaline proteases, amylase -Hydrolysis	Probe/ 20, 40 kHz/ 2.2 W/cm²	Photometry	US improves enzymes diffusion through the skin, with little effect on activity	[26]
Sewage sludge	Sludge flocs	Cells/ Enzymes	-Protease, phosphateses, α-amylase, α-glucosidase	Baths/20, 40 kHz	Photometry	US action: cell disruption, enzyme extraction	[44]
Fish	Fish bile	Endocrine-disrupting compounds	-β-Glucuronidase, β-glucosidase, sulfatase -Hydrolysis	Probe/ 20 kHz, 70W	Derivatization+ GC–MS	Shortening of hydrolysis time from 16 h to 20 min	[33]
Pollutants	Dairy wastewater	Fats	-Lipase -Hydrolysis	Bath/25, 40 kHz/165 W	Titration	Shortening of reaction time	[68]
Pollutants	Phosphate buffer	Cetirizine dihydrochloride	-Laccase -Oxidation	Bath/25 kHz/100 W	HPLC–UV, LC–MS	Shortening degradation time	[25]
Waste newspaper	Delignified Newspaper, citrate	Lignocellulose	-Cellulase -Hydrolysis	Probe/ 20 kHz/ 120 W	Photometry	Decrease of hydrolysis time and enzyme loading. Improvement of yield of reducing sugars.	[140]
Biodiesel byproduct	Tert-butyl alcohol	Glycerol, dimethyl carbonate	-Lipase -Transterification	Bath/25, 40 kHz/200 W	GC–FID	Shortening of processing time	[75]

Table 3. Applications of the US–enzyme binomial in the clinical area.

Type of study	Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device/ features	Analytical technique	Comments	Ref.
US effect on enzymes	Plasma	*o*-Nitro-phenyl-butyrate, butyrylthi-ocholine, aspirin	-Butyryl-cholines-terase	Probe/ 20 kHz	Gel electro-phoresis, photometry	Enzyme denaturation-inactivation by US	[8]
Remedia tion of US effects	Rat liver	Microsomal, cytosolic liver fractions	-Oxidases, cytochromo P450 -Oxidation	No data/2 W/cm²	Photometry	Inhibition by US, correction by heparin	[14]
Activation, degradation	Buffer	Chymotryps inogen, trypsinogen	-Chymo-trypsinogen, trypsinogen -Proteolysis	Probe/ 26.4 kHz/ 26 W/cm²	Photometry, HPLC–UV	US minimizes effects of trombolysis	[28]
Wheat	Protein hydrolyzate	ACE[a]-inhibitory activity	-Alcalase -Hydrolysis	Probe/ 20 kHz/ 40 W	IEC–FL[b], SEM	US increases enzymatic hydrolysis and ACE-inhibitory activity	[113]
Dopant drugs	Urine	Anabolic steroids	-β-Glucu-ronidase -Hydrolysis	Probe, reactor, bath (35, 130 kHz)	Derivati-zation+ GC–MS	US-assisted enzymatic hydrolysis and derivatization	[17]
Dopping control	Urine	Anabolic androgenic steroids	-β-Glucu-ronidase -Hydrolysis	Probe	HLPC–UV-visible	Study of kinetics parameters	[70]
Health-care foods	Epimedium leaves	Polysaccha-rides extract	-Papain, pectase, cellulase α-amylase -Hydrolysis	Bath/40 kHz/300 W	Ultrafiltra-tion, chroma-tography-DEAE-Sepharose, Photometry	US provides extracts with higher antioxidant power	[88]
Implants materials	ILs	Monomers	-Lipase -Polymeri-zation	Bath/35 kHz/320 W	NMR, GPC–RI[c]	Ring opening polymerization of ε-caprolactone monomer	[105]
Ester synthesis	ILs	Caffeic acid and metanol	-Lipases -Esterifica-tion	No data/ 15, 20, 25, 30, 35 kHz/ 90, 120, 150,180, 210 W	LC-DAD-MS, HPLC–UV-vis	US shortens reaction time and increases synthesis yield	[141]
Enzyme purification	Cells and fermenta-tive medium	Azoalbumin	-Fibrinolytic enzyme -Hydrolysis	Bath/25, 40 kHz /150W	Photometry	US-assisted three-phase partitioning to increase % recovery of fibrinolytic enzyme.	[125]
Enzyme production	Cells and fermenta-tive medium	Azoalbumin	-Fibrinolytic enzyme -Hydrolysis	Bath/25 kHz /160W	Photometry, SEM	US-assisted mass transfer on enzyme production	[123]
Enzyme production	Cells and fermenta-tive medium	Azoalbumin	-Fibrinolytic enzyme -Hydrolysis	Bath/25 kHz/ 160W	Photometry	US-assisted mass transfer on in-bioreactor enzyme production	[122]

[a]ACE, angiotensin I-converting enzyme; [b]IEC–FL, ion-exchange chromatography–fluorescence detection; [c]GPC–RI, gel permeation chromatography–refractive index detector.

Table 4. Applications of the US–enzyme binomial in the industry.

Area	Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device/ features	Analytical technique	Comments	Ref.
Textile	Cotton	Fibers	-Cellulase, peptinase -Hydrolysis	Reactor/ 16, 20 kHz/ 1.4 kW	Tensil forcers, wettability[a]	Shortening of processing time, less fiber damage, better uniformity of the treatment	[40]
Textile	Silk	Fiber	-Alcalase, savinase, and mixtures -Hydrolysis	Bath/70W	SEM	Degumming process. No silk deterioration by US	[21]
Textile	Micro-emulsions	2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	-Laccase -Oxidation	Bath/45 kHz/ 120 W	Photometry	US enhances stability of micro-emulsions of laccase enzymes	[27]
Textile, food	Potato	Starch	-α-Amyloglu-cosidase -Hydrolysis	Bath/40 kHz/ 132 W	Photometry	Different enzymes response to chemical/physical variables after US application	[53]
Textile, pharmacologi cal	Organic solvents	Amines	-Lipase -Alkylation	Probe/22 kHz/ 750 W/ 3.4×10^3 W/cm^2	FT-IR, NMR, Q-TOF	Despite the analytical instrumentation used, no information on US action is provided	[11]
Biodiesel	Organic solvent	Soybean oil	-Lipases -Transesterification	Bath/37 kHz/ 132 W	GC–FID	US increases biodiesel yield and shorts production time.	[54]
Biofuel	Bamboo powder, ILs	Cellulose	-Cellulase -Hydrolysis	Reactor/ 24 kHz/35W	SEM, PXRD	ILs+US-assisted pretreatment decreases cellulose crystallinity	[142]
Biofuel	Sugarcane bagasse, SCF[b]	Sugarcane	-Cellulase -Hydrolysis	Bath/40 kHz/ 154 W	Photometry	SCF+US-assisted pretreatment with drastic yield increase. Low temperatures, non-toxic solvents	[100]

Continuation Table 4

Area	Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device/ features	Analytical technique	Comments	Ref.
Biofuel	Waste cooking oil	Triglycerides	-Lipase -Transesterification	Bath/25 kHz/ 200 W	HPLC–UV	US-assistance for 4 h provides 86.61% yield	[86]
Biofuel	Soybean oil	Triglycerides	-Lipase -Transesterification	Bath/40 kHz/ 132 W	Titration	US-assistance+stirring for 2 h provided 95% yield	[85]
Bioethanol	Yam	Starch	-Amylase -Hydrolysis	Bath/ 40 kHz/ 132W	Photometry	US-assistance favor both enzymatic and chemical hydrolysis	[109]
Bioethanol	Citrate buffer	Carboxymethyl cellulose	-Cellulase -Hydrolysis	Probe/20 kHz/ 17.33 W cm^{-2}	Photometry	Increase the number of tryptophan on cellulase surface. Activation of enzyme activity.	[61]
Energy	Oil and ethanol	Fatty acids and ethanol	-Lipase -Esterification	Bath/40 kHz/ 132 W	GC–FID	Improved yields in short reaction times.	[143]
Food, feed, pulp, paper, textile and bioethanol industry	Solid-state fermentation cultures	Glucuronoxylan, *p*-nitrophenyl-β-D-glucopyra-noside, hydroxy-ethyl-cellulose, polygalacturonic acid, veratryl alcohol and phenol red.	-Endoxylanase, 1,4-β-D-glucosidase, 1,4-β-D-endo-glucanase, polygalacturonase, lignin peroxidase, manganese peroxidase, laccase	Reactor/20 kHz/ 500 W	Photometry	US increases extraction of the enzymes from the solid-state fermentation materials	[124]
Pharmaceu-tical	Non-matrix or 2-propanol	Methylbenzoate-glycerol	-Lipase -Transesterification	37 kHz/132 W	GC–FID, NMR	US-assisted maximization of conversion	[32]
Pharmaceu-tical, nutraceuti-cals	Organic solvent	Rutim, narangin	-Lipase -Esterification	Probe/ 20–40 kHz/ 50–250 W	HPLC–UV	US-assistance shortens to 24 h the UFAs esterification step	[55]

Continuation Table 4

Area	Sample matrix	Substrate or enzyme	-Enzyme -Catalyzed reaction	Type of US device/ features	Analytical technique	Comments	Ref.
Pharmaceu-ticals, food	Aqueous buffer	Dextran	-Dextranase -Hydrolysis	Probe/ 25 kHz/ 100 W	Photome-try, HPLC–RI	US-assistance increases dextranase activity, and tryptophan on the enzyme surface as compared with incubation at 50 ºC.	[144]
Pharmaceu-tical, cos-metics, food	Isoamyl alcohol, butyric acid	Isoamyl alcohol, butyric acid	-Lipase -Transesterification	Bath/ 25, 40 kHz/ 70 W	Titration	Shortening of reaction time.	[74]
Cosmetics, renewable energy, feed, organic chemicals, synthetic polymers	Wheat chaff	Lignocellulosic biomass	-Lignolytic enzymes from white rot fungal -Hydrolysis	Bath/40 kHz/ 0.5 W cm^{-2} Bath/400 kHz/ 0.5 W/cm^2	Photome-try, GC–MS	US+lignolytic enzyme increase degradation.	[145]
Cosmetics	Non edible oil	Fats or free fatty acids and methanol	-Lipase -Hydrolysis, esterification	Probe/20 kHz/ 50 W	Titration	US increases esterification yield and shortens the reaction time.	[87]
Pharmaceu-tical, cos-metics, food	Butyric acid, ethanol	Butyric acid, ethanol	-Lipase -Esterification	Bath/40 kHz/ 220 W	Titration	US+molecular sieves increase concentrations of substrates and enzyme activity.	[146]
Biosensors, biocatalysis.	Mesoporous silica	Enzyme (immobilization)	-Lysozyme	No data/ 45 kHz/ 400 W	Photome-try, XRD, SEM and TEM	US affects morphology and particle size of silica	[57]

[a]No analytical parameter; [b]SCF, supercritical fluid.

Table 1 is devoted to US–enzymes application in the food field, which is that has so far received more attention. In addition to the improved yields of products of great interest in processed foods by favoring enzyme activity (*e.g.,* mono- and diacylglycerols, polysaccharides, fermentable sugars, esterified and transesterified products) improvement of fresh food quality by delay or avoidance of fruit browning deserves also be stressed.

Table 2 summarized outstanding contributions to the environmental area in which a fast removal of the given pollutant is always desirable. Some of the applications of US–enzymes in the environmental field deal with the acceleration of sample preparation methods for subsequent determination of toxicants [10] or cosmetics [70], which constitute valuable contributions from the point of view of contaminants analyses.

Table 3 shows an overview of the varied applications of US–enzymes in the clinical field. Thus, from enzyme production and purification, through synthesis or extraction of compounds of clinical interest, to acceleration of sample preparation in dopping control, the effect of US on enzyme activation are of great interest; nevertheless, enzyme inactivation and/or degradation can even be more interesting. Remediation of US effects on living systems is also a matter of study for better understanding the damage US can exert on living materials.

Last, but not the least, are industrial applications of US–enzymes, to which Table 4 is devoted. In it, a trending topic as biofuel production is successfully assisted by the US–enzyme binomial. Traditional problems in the textile industry are also overcome or minimized by the use of the binomial, as do in the pharmaceutical and cosmetics industries.

11. Conclusions

The following aspects can be pinpointed from the study of the research so far developed on US–enzyme uses:

1. Especial care should be devoted to the nomenclature related to US and the way to express the variables and parameters involved in this type of energy. The metadata in this area can be of paramount importance when other scientists try to reproduce an experiment to work on the basis established by previous research as a way to advance in the knowledge.

2. The role of US frequencies on the effect caused by this energy as a function of matrix–analyte–enzyme has been poorly considered. The scant studies on this aspect allow evidencing an enormous field of possibilities, which should be solidly established.
3. Analytical equipment different from photometers and titrimeters is mandatory to know the real effect of US on the given target. Despite techniques such CD, SEM, FT-IR are starting to be used, not enough information is obtained, most times, from the data they provide.

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Metabolómica vegetal

Plant metabolomics

Section B encompasses the metabolomics research in the plant field developed by the PhD student. The three different topics addressed in this section open three interesting lines, which require further wide and in depth research.

As a consequence of the information handled to write Chapter III, the research in Chapter IV open the door to the enormous and unknown potential of the US–enzyme binomial. The enzymatic hydrolysis of oleuropein in olive leaf extracts to oleuropein aglycon was studied using a multivariate design in which different hydrolases showed different behavior for different values of US variables (*viz.*, duty cycle, amplitude, cycle time). The study of a key US variable (frequency) is a pending goal that requires the design of US devices that only differ in this variable and keep constant the others. Attempts to achieve these designs have been unsuccessful so far.

Among the healthy properties of mushrooms, the recently described "*in vitro*" inhibition of the protease NS3 of hepatitis-C virus (HCV) by aqueous enzymatic extracts of the common edible mushroom *Agaricus bisporus* (*A. bisporus*) promoted interest in an untargeted study on the components of these extracts. Chapter V contains the results obtained by a liquid chromatography–tandem mass spectrometry (LC–QTOF MS/MS) platform working in high resolution mode: 55 out of 123 total metabolites tentatively investigated by combination of MS and MS/MS information were identified for the first time; a representative snapshot of the composition of the target extracts.

The increased legal pharmacological uses of cannabis and the implementation of breeding programs with agronomical purposes have made mandatory a wide study of this plant by gas chromatography–time-of-flight/mass spectrometry (GC–TOF/MS) and LC–QTOF MS/MS) in high resolution mode. The identified compounds in the extracts were 169, as shows Chapter VI, and the difference in their concentrations as a function of the in field or in greenhouse cultivation open a door to a more rational grown of this plant as required.

Chapter IV

Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts

Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts

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Abstract

Hydrolysis of oleuropein, the main phenol in olive (*Olea europaea L.*) leaf extracts, to oleuropein aglycon and other subsequent products in the hydrolytic pathway can be catalyzed by different enzymes. Three of the most used hydrolases were assayed to catalyze the process, and *β*-glucosidase from *Aspergillus niger* was selected. Acceleration of the enzymatic hydrolysis by ultrasound (US) was studied using a Box-Behnken design (duty cycle, amplitude, cycle time) and an oleuropein standard, and the optimum US conditions for achieving maximum yield of oleuropein aglycon were 0.5 s/s duty cycle, 50% amplitude and 45 s cycle. The method was applied to obtain oleuropein aglycon from commercial and laboratory extracts from olive leaves, which may have a pharmacological use as deduced by its healthy properties. The kinetics of the US-assisted enzymatic hydrolysis was monitored by analysis of the target compounds using liquid chromatography–tandem mass spectrometry.

Keywords: *e*nzymatic hydrolysis, ultrasound, *Olea europaea* leaf extract, glucosidase, oleuropein, oleuropein aglycon.

1. Introduction

Oleuropein and oleuropein aglycon, present in virgin olive oil (VOO), could be used as nutraceutical or as component of new functional foods (Van der Stelt *et al.*, 2015). Oleuropein and its aglycon possess beneficial pharmacological properties such as cardioprotective (Manna *et al.*, 2004), antioxidant (Andreadou *et al.*, 2006), anti-inflammatory (Khalatbary & Zarrinjoei, 2012), anti-cancer (Hamdi & Castellon, 2005), and neuroprotective effects (Bazoti, Bergquist, Markides & Tsarbopoulos, 2006), among the most important.

The use of VOO as a source of oleuropein and/or oleuropein aglycon for preparation of functional foods is not recommended owing to the high price of VOO and relative low concentration of these minor components. An almost ideal raw material for extraction of these and other valuable compounds are olive leaves: a residue generated from olive pruning and VOO production.

Olive leaves may account up to 5% of the weight of collected olives and are removed in the step of cleaning olive drupes in the oil mill prior to VOO production (Zoiopoulos, 1983). By sum up the leaves from this step to those from pruning olive trees, the total amount of twigs and leaves residues may reach 12–30 kg/tree (Nefzaoui, Hellings & Vanbelle, 1983). There are almost 900 million olive trees over 10 million hectares worldwide, 98% of which in the Mediterranean countries (Sesli & Yegenoglu, 2009); therefore, the management of these residues should be properly exploited taking into account the high valuable compounds they contain (Xie, Huang, Zhang & Zhang, 2016), and the scant applications developed so far. The present main uses of olive leaves are animal feed, production of thermal energy, extraction of useful components (Joint Ministerial Decision, 2012), or composting (the last probably because the high C/N ratio of this residue) (Manios, 2004).

The aglycon form of oleuropein is obtained by enzymatic hydrolysis of oleuropein, being β-glucosidase the key enzyme involved in the process, as shows Fig. S1, which also shows the possible subsequent evolution of oleuropein aglycon to hydroxytyrosol and/or decarboxymethyloleuropein aglycon depending on the working conditions.

The different sources of β-glucosidase to catalyze the hydrolysis of oleuropein to obtain hydroxytyrosol and/or oleuropein aglycon have been almonds (Briante *et al.*, 2000; Jemai, Bouaziz, Fki, Feki & Sayadi, 2008), *Sulfolobus solfatarius* (Briante *et al.*, 2000;

Briante, La Cara, Febbraio, Patumi & Nucci, 2002; Briante, Patumi, Febbraio & Nucci, 2004), or *Aspergillus niger* (Hamza & Sayadi, 2015; Khoufi, Hamza & Sayadi, 2011), which require intervals from 2 to 16 h to complete hydrolysis (Briante *et al.*, 2004; Jemai *et al.*, 2008). Also hemicellulase, a glycolytic enzyme that decomposes hemicellulose of plant cell walls, has been proposed to hydrolyze oleuropein, to hydroxytyrosol in this case (De Faveri, Aliakbarian, Avogadro, Perego & Converti, 2008; Yuan, Wang, Ye, Tao & Zhang, 2015).

Ultrasound (US) as modifier of enzyme activity has provided very controversial results (Delgado-Povedano & Luque de Castro, 2014). While oxidases seem to suffer deactivation under US application, an activity enhancement on hydrolytic enzymes is experienced in the case of sugarcane bagasse for the production of fermentable sugars, with yield of 0.26 g sugar/g dry sugarcane bagasse, around twice the value obtained without US assistance (Lunelli *et al.*, 2014). Also in the enzymatic hydrolysis for extraction of luteolin and apigenin from celery, US-assistance provided yield increases of 26.1-fold and 32.2-fold, respectively, as compared with no ultrasonicated systems (Zhang, Zhou, Chen, Cao & Tan, 2011).

With these premises, and with the aim of developing a fast method for enzymatic hydrolysis of oleuropein to its aglycon with minimum production of more degraded products, the following steps were planned: (i) selection of the most suitable hydrolase among the three more frequently used in the literature; (ii) application of US to shorten the enzymatic hydrolysis step as much as possible; (iii) use of a Box-Behnken design (BBD) to optimize the US variables duty cycle, amplitude, and cycle time for maximum acceleration of the hydrolysis of an oleuropein standard; (iv) application of the method to different olive leaf extracts.

2. Materials and methods

2.1. Olive leaf samples

Olive leaves collected from January to March 2015 from different cultivars located in the Denomination of Origin “Baena” in the South of Spain were used to obtain phenols extracts.

Commercial olive leaf extract powder enriched in oleuropein (40% oleuropein), from Ferrer HealthTech (Barcelona, Spain), was also used in this work for comparison with the behavior of fresh extracts.

2.2. Chemicals and reagents

Standards of oleuropein (purity ≥90%) and hydroxytyrosol (purity >98.0%) for quantitative analysis of these metabolites were from Extrasynthese (Genay, France). Standards of oleuropein aglycon, decarboxymethyl oleuropein aglycon, decarboxymethyl ligstroside aglycon and ligstroside aglycon were donated by P. Magiatis (Athens, Greece). The three tested enzymes (hemicellulase and *β*-glucosidase from *A. niger,* and *β*-glucosidase from almonds, the characteristics of which are listed in Table S1) were all from Sigma–Aldrich (Madrid, Spain). MS (mass spectrometry) grade glacial acetic acid from Scharlab (Barcelona, Spain) and anhydrous sodium acetate from Panreac (Barcelona, Spain) were used to prepare buffers at different pHs. MS grade formic acid (FA) from Scharlab and methanol from Sigma–Aldrich were used to prepare the chromatographic mobile phases. Methanol was also used for sample preparation. Deionized water (18 mΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare aqueous solutions.

2.3. Extraction of oleuropein from olive leaves

Olive leaves (1 g) were dried and chopped, and 30 mL of 4:1 (v/v) methanol–water was added to the chopped olive leaves and the heterogeneous system thus formed was kept under agitation for 24 h in a Vibromatic rocking mixer from JP Selecta S.A. (Barcelona, Spain) for transfer of the polar compounds to the liquid phase. Then, the system was centrifuged at 1500×*g* for 15 min, the extract was filtered through 0.2 μm filter and divided into 8 aliquots before evaporation to dryness in a Concentrator Plus speed-vac from Eppendorf (Hamburg, Germany). This approach has been widely validated in previous research and it is accepted as sample preparation strategy prior to quantitative analysis of oleuropein in olive leaves (Jemai *et al.*, 2008).

2.4. Enzymatic hydrolysis

The hydrolysis step catalyzed by each of the three enzymes under study was performed by locating 10 mg of oleuropein in each of three 12-mL glass bottles, and adding to them the same units (2.5 U/mg oleuropein substrate) of one of the enzymes and 10 mL of 0.05 M sodium acetate buffer. The amount of enzyme to be added depended on its activity: 21.6 mg of *β*-glucosidase from *A. niger* (1.2 U/mg), 3.5 mg of *β*-glucosidase from almonds (7.7 U/mg) and 16.7 mg of hemicellulase from *A. niger* (1.5 U/mg). The bottles were placed in a digital block heater, model SBH130D, from Stuart (Staffordshire, ST15 OSA, UK) to maintain constant temperature during the enzymatic hydrolysis. The enzyme–substrate ratio had been optimized in a previous study (Yuan *et al.*, 2015), and other enzymatic conditions (the best temperature and pH) for each enzyme were those commercially recommended (37 ºC for both *β*-glucosidases and 40 ºC for hemicellulase, and pH 4.0 for *β*-glucosidase from *A. niger*, pH 5.0 for *β*-glucosidase from almonds and pH 4.5 for hemicellulase).

Sampling at different enzymatic hydrolysis times (0, 0.5, 2, 4, 6, 8, 10 and 12 h) was performed. The samples were immediately frozen and stored at −20 ºC to stop the catalyzed reaction (Molinari & Silva, 1955), then analyzed to monitor the development of the reaction at each preset time. Also the evolution of the no catalyzed reaction was tested by developing the experiment in the absence of biocatalyst.

The hydrolysis of oleuropein was calculated by the following expression: [(concentration of oleuropein in the sample before hydrolysis – concentration after hydrolysis)/concentration in the sample before hydrolysis] × 100.

2.5. US-assisted enzymatic hydrolysis (USAEH) of oleuropein standard

USAEH of oleuropein by *β*-glucosidase from *A. niger* was performed under the conditions described in section 2.4. The mixture was immersed in a water bath to maintain constant the temperature during hydrolysis. The ultrasonic probe —a Branson 450 digital sonifier (20 kHz, 400 W) with tunable amplitude, corresponding 100% of amplitude to 400 W, and duty cycle, equipped with a cylindrical titanium alloy probe microtip (3 mm in diameter)— was dipped into the solution, with the microtip horn at a fixed distance from the bottom (2 mm) of the container without contacting it. The probe was totally immersed

into the solution to avoid formation of aerosol and foam that create dead zones where cavitation does not occur. A water-bath from Selecta (Barcelona, Spain) with a cooling system was used to maintain the temperature constant during USAEH. The US conditions were a 0.5 s/s duty cycle, 50% amplitude and 45 s cycle time. A 30 s off interval separated two consecutive US cycles. Sampling was performed after 0, 10, 15, 20, 25, 30, 45 and 60 consecutive US cycles, and the samples were frozen (−20 ºC) until analysis. Also the evolution of the no catalyzed reaction (in the absence of biocatalyst) was monitored.

2.6. USAEH of olive leaf extracts

Hydrolysis of oleuropein in the commercial olive leaf extract was carried out by taking 25 mg of it (equivalent to 10 mg of oleuropein), putting it into contact with 10 mL of 0.05 M sodium acetate buffer (pH 4) and the appropriate amount of the selected enzyme (2.5 U/mg oleuropein substrate).

For hydrolysis of oleuropein in the extract prepared as described in Section 2.3, the solid residue generated after dryness of one of the aliquots was put into contact with 10 mL of 0.05 M sodium acetate buffer (pH 4) and the appropriate amount of the selected enzyme (2.5 U/mg oleuropein substrate).

Both systems were subjected to US under the conditions described in section 2.5 and the evolution of the reaction was monitored by analyzing the samples taken at preset number of consecutive US cycles.

2.7. LC–MS/MS analysis

An Agilent (Palo Alto, CA, USA) 1200 Series liquid chromatography (LC) system (consisting of a binary pump, a vacuum degasser, an autosampler and thermostated column compartment) was used for chromatographic separation, and an Agilent 6460 triple quadrupole mass spectrometer equipped with a Jetstream® electrospray ionization (ESI) source was used for detection. The data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis.

The analytical column was a reverse phase 5 μm particle size, 150 mm×4.6 mm i.d. C_{18} Mediterranean Sea from Teknokroma (Barcelona, Spain), which was thermostated at

30 ºC. A pre-column, 40 mm×3.0 mm i.d., from Phenomenex (Torrance, CA, USA) was connected to the analytical column for protection.

All samples were 1:500 diluted with 60:40 (v/v) methanol–water before analysis. The mobile phases were: 0.1% FA in water (phase A) and 0.1% FA in methanol (phase B). The LC pump worked at a constant flow rate of 0.8 mL/min with the following elution gradient: 20% phase B for 1 min, after which a linear gradient for 5 min was programmed up to 66% phase B. Then, a second gradient up to 100% B for 7 min was applied and, finally, 100% B for 2 min. A post-time of 5 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 10 µL and the injector needle was washed for 12 s between injections with 60% methanol to avoid cross contamination.

MS detection was performed in the negative ESI mode at unit resolution in both quadrupoles. The Jetstream ESI source parameters, operating in the negative ionization mode, were as follows: nozzle and capillary voltage were set at 0.5 kV and 3 kV, respectively, N_2 nebulizer was flowed at 50 psi, N_2 drying gas flow rate and temperature were 10 L/min and 300 ºC, N_2 sheat gas flow rate and temperature were 12 L/min and 360 ºC. The selected voltage for efficient filtration of precursor ions in the first quadrupole, collision energy to fragment the precursor ions by collision-induced dissociation to generate the product ions selected as quantitation and qualifier ions in the selected reaction monitoring (SRM) method for each analyte are shown in Table 1. The dwell time was fixed at 200 ms for each SRM transition. Identification and quantitation of the target compounds were performed using pure standards for all monitored compounds. All samples were injected in duplicate.

Table 1. MS/MS parameters for qualitative and quantitative determination of the target compounds.

Compound	Fragmentor (V)	Precursor ion (*m/z*)	Collision energy (eV)	Quantitation transition	Product ion confirmation (*m/z*)
Oleuropein	170	539.2	23	539.2 → 307.1	275.0
Oleuropein aglycon	110	377.1	12	377.1 → 275.1	307.0
Ligstroside aglycon	110	361.1	12	361.1 → 291.1	259.0
Decarboxymethyl oleuropein aglycon	110	319.1	12	319.1 → 59.0	123.0
Decarboxymethyl ligstroside aglycon	110	303.1	12	303.1 → 59.1	137.0
Hydroxytyrosol	110	153.0	12	153.0 → 123.0	108.0

2.8. SRM-based quantitation

Quantitative analysis of the target compounds in the olive leaf extracts was carried out using the peak area from the transition of each compound, which coincided with the chromatographic retention time of the standard. Calibration models were developed for each compound using multistandard solutions at seven concentrations that depended on the target analyte. All calibration points were obtained in duplicate injection to set the confidence intervals. The calibration ranges and regression coefficients are shown in Table 2. As can be seen, the regression coefficients were above 0.998 for all analytes, and the upper limits for the calibration models depended on the target compound. The limits of detection (LOD) and quantitation (LOQ) of the method for each target compound were calculated as the concentration providing signals three and ten times, respectively, higher than the background noise in the elution window close to each peak. They were between 0.1 ng/mL and 0.24 μg/mL for LODs and from 0.3 ng/mL to 0.75 μg/mL for LOQs.

Table 2. Linear calibration ranges, calibration equations, regression coefficients and limits of quantitation for analysis of the target compounds.

Compound	Linear calibration range (μg/mL)	Calibration equation	Regression coefficient (R^2)	LOD (ng/mL)	LOQ (ng/mL)
Oleuropein	0.01–10	y = 13034.31x +12.93	0.9990	3.0	10.0
Oleuropein aglycon	$3{\cdot}10^{-4}$–10	y = 10678x +219.66	0.9998	0.1	0.3
Ligstroside aglycon	$3{\cdot}10^{-4}$–0.20	y = 33044x +42.72	0.9992	0.1	0.3
Decarboxymethyl oleuropein aglycon	$3{\cdot}10^{-4}$–1	y = 10354x +34.21	0.9997	0.1	0.3
Decarboxymethyl ligstroside aglycon	$1.7{\cdot}10^{-3}$–5	y = 4393.76x +166.14	0.9998	0.5	1.7
Hydroxytyrosol	$3{\cdot}10^{-4}$–1	y = 44321x +72.46	1.0000	0.1	0.3

y: peak area; x: concentration as μg/mL.

3. Results and discussion

3.1. Enzyme selection for hydrolysis of oleuropein

The most used biocatalysts to hydrolyze oleuropein to oleuropein aglycon —*viz.*, *β*-glucosidase from almonds (Briante *et al.*, 2000; Jemai *et al.*, 2008), *β*-glucosidase from *A. niger* (Hamza *et al.*, 2015; Khoufi *et al.*, 2011), and hemicellulase from *A niger* (De Faveri *et al.*, 2008; Yuan *et al.*, 2015)— were assayed in this comparative study under the conditions recommended by the manufacturer. Fig. 1 shows that complete hydrolysis of oleuropein was achieved by *β*-glucosidase from *A. niger* and from almonds. The highest catalytic effect was provided by *β*-glucosidase from *A. niger* as complete hydrolysis was achieved within 2 h, while the same enzyme from almonds required 4 h for complete hydrolysis, and hemicellulase from *A. niger* provided a low catalytic efficiency that did not surpass 30%.

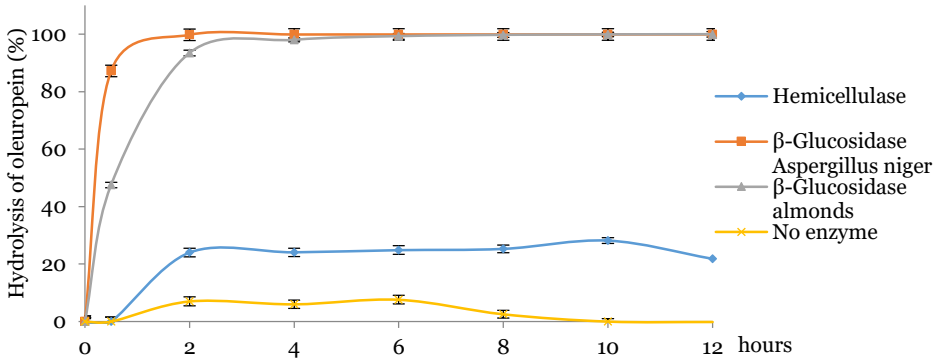

Fig. 1. Enzymatic effect of different enzymes on the hydrolysis of an oleuropein standard (10 μg/mL) as compared with the no catalyzed reaction.

These results are not in agreement with those in a previous work in which different enzymes were compared, and where hemicellulase gave the best catalytic behavior, but in which the hydrolysis was optimized to obtain hydroxytyrosol as the final product (Yuan *et al.*, 2015).

In the present research, and for all the enzymes tested, the main product from hydrolysis of oleuropein was oleuropein aglycon, with small generation of other hydrolysis products depending on the target biocatalyst, as shows Fig. S2. Thus, decarboxymethyl oleuropein aglycon and hydroxytyrosol were also formed by *β*-glucosidase from *A. niger* (see Fig. S2A). Oleuropein aglycon increased from 0 to 2 h and then decreased linearly up to 12 h, while decarboxymethyl oleuropein aglycon and hydroxytyrosol slightly increased from 0 to 12 h, but the final amounts of them were 0.06 μg/mL and 0.04 μg/mL, respectively. When *β*-glucosidase from almonds was used the main hydrolysis product was oleuropein aglycon, which increased from 0 to 6 h and kept constant from 6 to 12 h. The other hydrolysis product was hydroxytyrosol, which increased slightly from 0 to 12 h, but the total amount formed did not surpass 0.04 μg/mL (Fig. S2B). When hemicellulase from *A. niger* was used the main hydrolysis products were oleuropein aglycon and hydroxytyrosol; both products increased from 0 to 12 h, but the total amounts formed were 0.25 μg/mL and 0.01 μg/mL, respectively (Fig. S2C).

With these results, *β*-glucosidase from *A. niger* was selected among the three enzymes tested for further studies since hydrolysis of oleuropein was complete within 2 h with not significant development of subsequent hydrolysis steps. It is worth mentioning that the hydrolysis of oleuropein in the absence of enzyme did not progress along the 12 h of the study (see Fig. S2D), thus proving the crucial role of the enzyme.

3.2. Effect of temperature on the enzymatic hydrolysis

The influence of temperature on the catalytic effect of *β*-glucosidase from *A. niger* in the hydrolysis of oleuropein was studied at the recommended temperature (37 ºC), and at lower (25 ºC) and upper (50 ºC) temperatures. As compared to the behavior at 37 ºC (complete hydrolysis within 2 h, see Fig. S2A) the hydrolysis of oleuropein at 25 ºC was complete after 8 h (see Fig. S3A). Therefore, the decrease of temperature also decreased the reaction kinetics as the hydrolysis pathway seemed to be the same because the coincidence of the hydrolysis product. On the other hand, the hydrolytic process was accelerated by increasing the temperature up to 50 ºC, and it was complete after 0.5 h (see Fig. S3B). However, in this case the hydrolysis progressed with formation of hydroxytyrosol as one of the main reaction products together with oleuropein aglycon (21% hydroxytyrosol, as compared with 3% formed after 12 h at 37 ºC). Additionally, other reaction products such

as decarboxymethyl ligstroside and decarboxymethyl oleuropein aglycon were detected when working at 50 ºC, with final amounts of 0.01 μg/mL for decarboxymethyl ligstroside aglycon and 0.01 μg/mL for decarboxymethyl oleuropein aglycon; therefore, a loss of selectivity occurred regarding the pursued oleuropein aglycon.

3.3. Optimization of USAEH

As acceleration of enzymatic hydrolysis by US is well documented (Delgado-Povedano *et al.*, 2014), the use of this energy to obtain the target aglycon in a time as short as possible without subsequent degradation was assayed by using a multivariate design and oleuropein standard. A 15-run BBD was used to optimize three interrelated US variables: X_1 (duty cycle), X_2 (amplitude) and X_3 (cycle time). Table S2 shows the input parameters and experimental design levels used. Different combinations of these US parameters were investigated, as shows Table S3 where the values of the dependent variable (formation of oleuropein aglycon and hydrolysis of oleuropein expressed as the concentrations ratio between the former and the latter) are also shown.

The oleuropein aglycon obtained at various levels of the three independent variables (X_1, X_2, and X_3) was subjected to multiple regression to yield a second-order polynomial equation, as follows:

$$Y = 2.70532 - 8.88722X_1 - 10.3086X_2 + 0.00486972X_3 + 15.3162X_1X_2 + 0.0485667X_1X_3 + 0.0703792X_2X_3 + 7.97026X_1^2 + 10.3218\,X_2^2 - 0.000410231X_3^2$$

where Y represents the ratio between the concentrations of oleuropein aglycon and oleuropein, and X_1, X_2 and X_3 represent duty cycle, amplitude and cycle time, respectively. According to the regression model equations, the fitting coefficient of the three variables provided values of 10.3086 > 8.88722 > 0.00486972, thus implying that X_1 (duty cycle) and X_2 (amplitude) were the most influential US variables.

The statistical parameters obtained from the analysis of variance are given in Table S4. The value of the determination coefficient (R^2 = 0.9225) and the adjusted determination coefficient (R_{adj}^2 = 0.8877) also confirmed the statistical significance of the model. In conclusion, the model was found to be adequate for navigating the design.

The *p*-value is used as a tool to check the significance of each coefficient, which in turn indicates the strength of the interaction among independent variables. It could be

observed that the coefficients of X_1, X_2, X_3, X_1X_2 and X_2^2 were highly significant (p<0.001); while the coefficient of X_1X_3, X_3^2 was found non-significant (p>0.05). The X_1, X_2 and X_3 variables had a significant effect on US. For the X_1X_2 interaction, p<0.001 implies that the duty cycle was closely related to amplitude in the US-assisted process.

The formation of oleuropein aglycon increased when the duty cycle was extended from 0.1 to 0.5 s/s, with the logical simultaneous decrease of oleuropein. The former also increased by increasing both the amplitude (from 10 to 50%) and the cycle time (from 15 to 45 s). The optimal US conditions to achieve maximum amount of oleuropein aglycon were duty cycle 0.5 s/s, amplitude 50% and cycle time 45 s. The amount of oleuropein aglycon obtained under the optimum conditions and applying 10 US cycles was 73.27±0.72% (n = 2). Values of the three interrelated variables higher than those within the BBD design were not tested because the temperature generated under these conditions was difficult to control when exceeding 37 °C, and could damage the microprobe and/or degrade the target aglycon.

A kinetics study was made testing different US cycles to determine the time necessary for total hydrolysis of the oleuropein standard. Each sample was ultrasonicated under the optimum working conditions (50% amplitude, 0.5 s/s duty cycle and 45 s cycle) at different number of US cycles within 0–60. The hydrolysis efficiency was calculated as the ratio between the area of the chromatographic peak provided by oleuropein aglycon obtained by USAEH and that obtained under identical working conditions but in the absence of US.

In addition to oleuropein aglycon, a small proportion of hydroxytyrosol was produced in the enzymatic hydrolysis of oleuropein in the presence of US. The formation of oleuropein aglycon increased from 0 to 10 cycles and decreased from 10 to 60 cycles, while hydroxytyrosol increased from 0 to 60 cycles, but the final amount of the latter was 0.01 μg/mL. After 10 cycles, subsequent hydrolysis of oleuropein to the aglycon form did not compensate owing to degradation to the subsequent product. During all the process time, the total number of moles was preserved.

An additional experiment to prove the effect of US on the enzymatic hydrolysis consisted of application of US under the optimum operational conditions in the absence of enzyme: no bond-breaking took place in the absence of enzyme as only oleuropein was found after the experiment.

Comparing the results obtained in the presence and absence of US, a drastic shortening of the time (from 2 h to 18.75 min with 15 US cycles) required for enzymatic hydrolysis of oleuropein was obtained with the help of US.

3.4. USAEH of olive leaf extracts

The usefulness of the proposed method was demonstrated by application to olive leaf extracts. The enzymatic hydrolysis with US assistance of oleuropein from a commercial extract from olive leaves yielded oleuropein aglycon and then hydroxytyrosol; but also decarboxymethyl oleuropein and ligstroside, present in the extracts in a smaller proportion due to the enrichment in oleuropein, were hydrolyzed to the corresponding aglycons. The hydrolysis yield of oleuropein was 90.8% after 10 US cycles, which produced 90% of oleuropein aglycon. The latter increased from 0 to 10 cycles and decreased very slowly from 10 to 60 cycles (final concentration at 60 cycles 82%). Ligstroside aglycon increased from 0 to 10 cycles and then decreased, its concentration being 0.03 μg/mL after 60 cycles. Decarboxymethyl oleuropein aglycon and hydroxytyrosol increased from 0 to 60 cycles and their concentrations were 0.026 and 0.031 μg/mL, respectively, after 60 cycles. Comparing the results with those obtained by hydrolysis of the oleuropein standard, it is clear that the sample matrix did not influence the results, as shows Fig. S4.

When working with olive leaf extracts prepared as described in Section 2.3, the products were the same as from commercial extracts. The hydrolysis yield of oleuropein reached 95.7% at 10 US cycles, with formation of 94.9% of the aglycon form. Fig. 2 shows the oleuropein-aglycon/oleuropein ratio (expressed as concentration) obtained from the three type of samples: oleuropein standard, commercial olive leaf extract and no commercial olive leaf extract. These results show that the behavior of oleuropein and that of the oleuropein aglycon was always the same, independent of the sample from which they come. As can be seen in Fig. 2, high statistical differences were seen for 15 US cycles (p-values<0.0055) in the three types of samples.

4. Conclusions

A USAEH method has been developed to obtain oleuropein aglycon from oleuropein in olive leaf extracts. β-Glucosidase from *A. niger* was selected among three

enzymes. A drastic shortening of the time required for complete hydrolysis was achieved as compared with a traditional method based on enzymatic incubation, which requires 2 h. Extraction of oleuropein from olive leaf extracts was carried out by agitation with a methanol–water mixture, while hydrolysis was carried out by *β*-glucosidase from *A. niger* and quantitation was by LC–MS/MS with multiple reaction monitoring. After multivariate optimization of the variables involved in the USAEH method, a kinetics study showed that the hydrolysis reaction of oleuropein was complete in only 18.75 min.

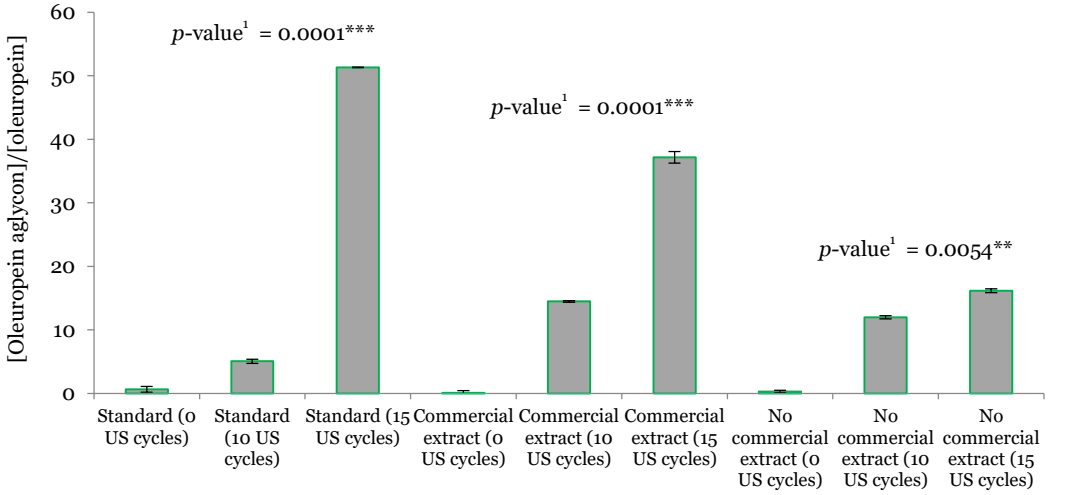

Fig. 2. USAEH (0–15 cycles) of oleuropein from an analytical standard (10 μg/mL), a commercial olive leaf extract and a no commercial olive leaf extract. The comparison to confirm statistical differences was based on ANOVA. [1]*p*-values were obtained by analysis of variance (ANOVA). ***p*-value between 0.001 and 0.01. ****p*-value < 0.001.

The fast production of oleuropein aglycon thus achieved, together with the fast methods for the extraction of phenols from olive leaves based on US or microwaves assistance previously reported by the authors (Japón-Luján, Luque-Rodríguez & Luque de Castro, 2006a; Japón-Luján, Luque-Rodríguez & Luque de Castro, 2006b), open a door to the exploitation of the interesting pharmacological properties of this compound (Casamenti, Grossi, Rigacci, Pantano, Luccarini & Stefani, 2015; Corominas-Faja *et al.*, 2014; Grossi *et al.*, 2013; Luccarini, Ed Dami, Grossi, Rigacci, Stefani & Casamenti, 2014), and its wide use for cosmetics, functional foods and nutraceuticals (Van der Stelt *et al.*, 2015).

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Supplementary material

Table S1. Specific information about the different enzymes.

Enzyme	pH	Temperature (ºC)	Enzyme activity (U/mg)	Enzyme form
β-Glucosidase from *Aspergillus niger*	4.0	37	1.2	Powder
β-Glucosidase from almonds	5.0	37	7.7	Lyophilized powder
Hemicellulase from *Aspergillus niger*	4.5	40	1.5	Powder

Table S2. Independent variables and their levels of Box-Behnken design for the hydrolysis reaction.

Independent variable	Symbol	Variable levels		
		-1	0	1
Duty cycle (s/s)	X_1	0.1	0.3	0.5
Amplitude (%)	X_2	10	30	50
Cycle time (s)	X_3	15	30	45

Table S3. Box-Behnken experimental design and corresponding oleuropein peak area.

No.	X_1 duty cycle (s/s)	X_2 amplitude (%)	X_3 cycle time (s)	Y [oleuropein aglycon]/ [oleuropein]
1	1	0	−1	0.87
1	1	0	−1	0.88
2	0	0	0	0.82
2	0	0	0	0.82
3	0	−1	1	0.83
3	0	−1	1	0.84
4	0	−1	−1	0.70
4	0	−1	−1	0.70
5	1	−1	0	0.87

Continuation Table S3

5	1	−1	0	0.88
6	0	0	0	0.79
6	0	0	0	0.78
7	−1	0	−1	0.82
7	−1	0	−1	0.82
8	−1	0	1	0.92
8	−1	0	1	0.92
9	0	1	−1	1.03
9	0	1	−1	1.01
10	−1	−1	0	0.90
10	−1	−1	0	0.89
11	−1	1	0	1.00
11	−1	1	0	1.00
12	0	1	1	2.01
12	0	1	1	1.99
13	1	0	1	1.55
13	1	0	1	1.58
14	1	1	0	3.46
14	1	1	0	3.40
15	0	0	0	0.85
15	0	0	0	0.85

Table S4. Variance analysis of items in the regression equation.

Sources of variation	**Sum of squares**	***df*[1]**	**Mean square**	***F* value**	***P* value**
$X_{1\ duty}$	2.42565	1	2.42565	41.60	0.0000
$X_{2\ am}$	4.29557	1	4.29557	73.67	0.0000
$X_{3\ cycl}$	0.91465	1	0.91465	15.69	0.0008
X_1X_2	3.00272	1	3.00272	51.50	0.0000
X_1X_3	0.16983	1	0.16983	2.91	0.1042

Continuation Table S4

X_2X_3	0.35663	1	0.35663	6.12	0.0230
X_1^2	0.75057	1	0.75057	12.87	0.0020
X_2^2	1.25881	1	1.25881	21.59	0.0002
X_3^2	0.06291	1	0.06291	1.08	0.3119
R^2	0.9225				
R_{adj}^2	0.8877				

[1] degree of freedom.

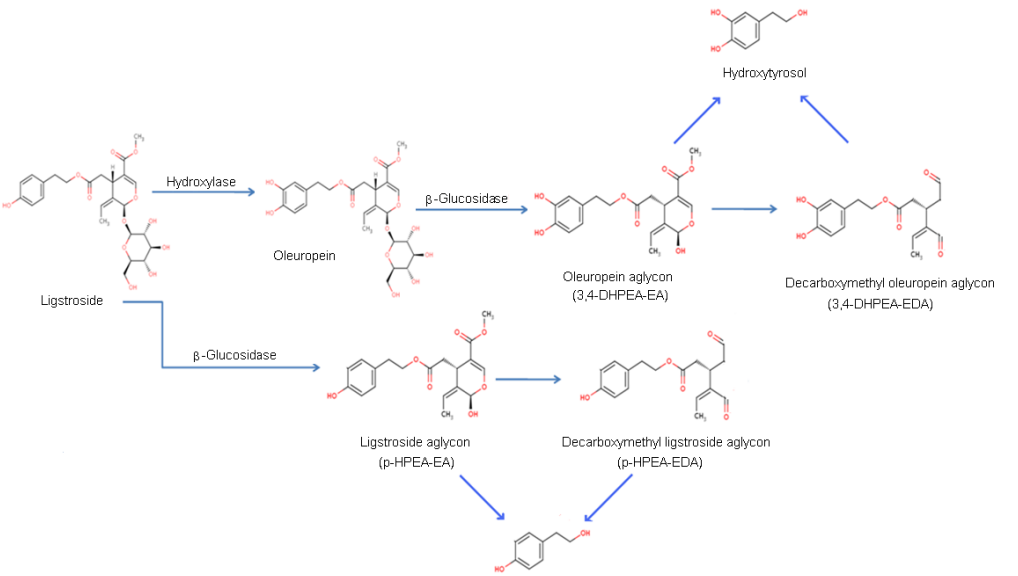

Fig. S1. Enzymatic hydrolysis of oleuropein.

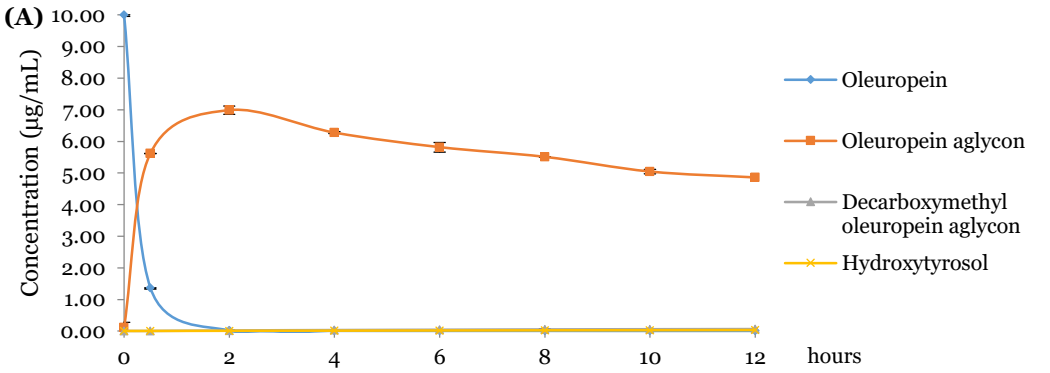

Fig. S2. (A) Hydrolysis of oleuropein to oleuropein aglycon and other minority products from an oleuropein standard (10 µg/mL) in the presence of β-glucosidase from *Aspergillus niger*.

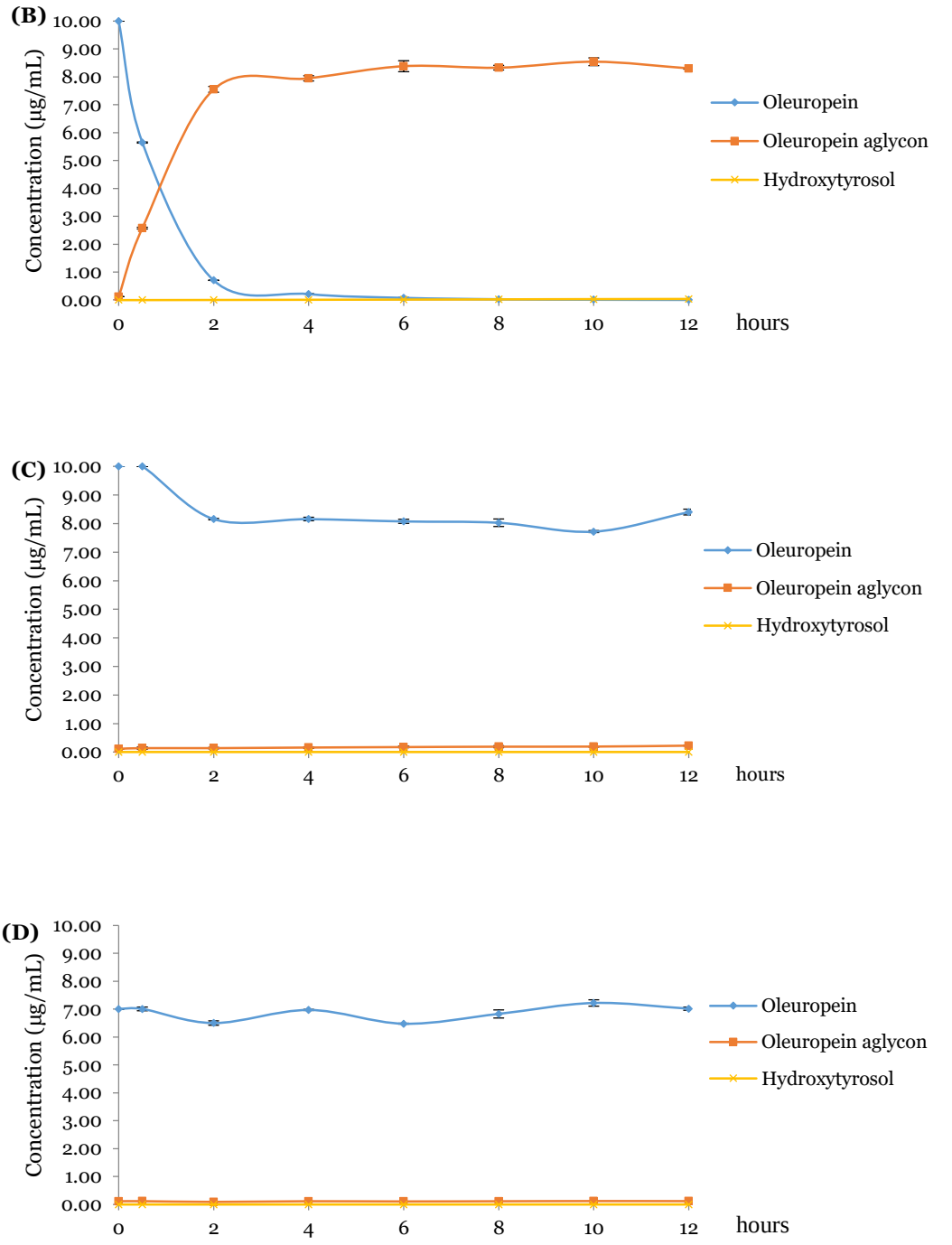

Fig. S2. Hydrolysis of oleuropein to oleuropein aglycon and other minority products from an oleuropein standard (10 µg/mL) in the presence of β-glucosidase from almonds **(B)**, hemicellulase from *Aspergillus niger* **(C)**, and the no catalyzed reaction **(D)**.

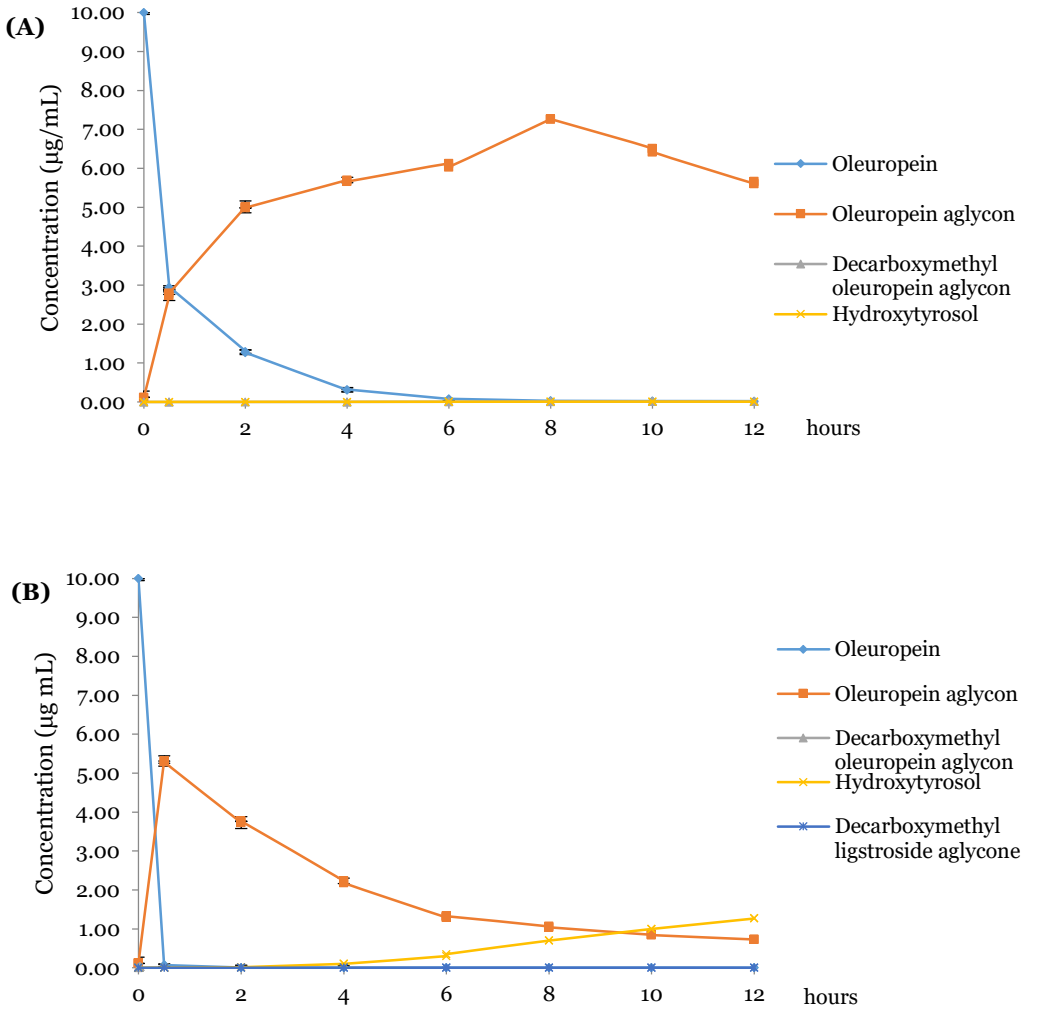

Fig. S3. Hydrolysis of oleuropein to oleuropein aglycon and other products from an oleuropein standard (10 µg/mL) in the presence of *β*-glucosidase from *Aspergillus niger* at 25 **(A)** and 50 ºC **(B)**.

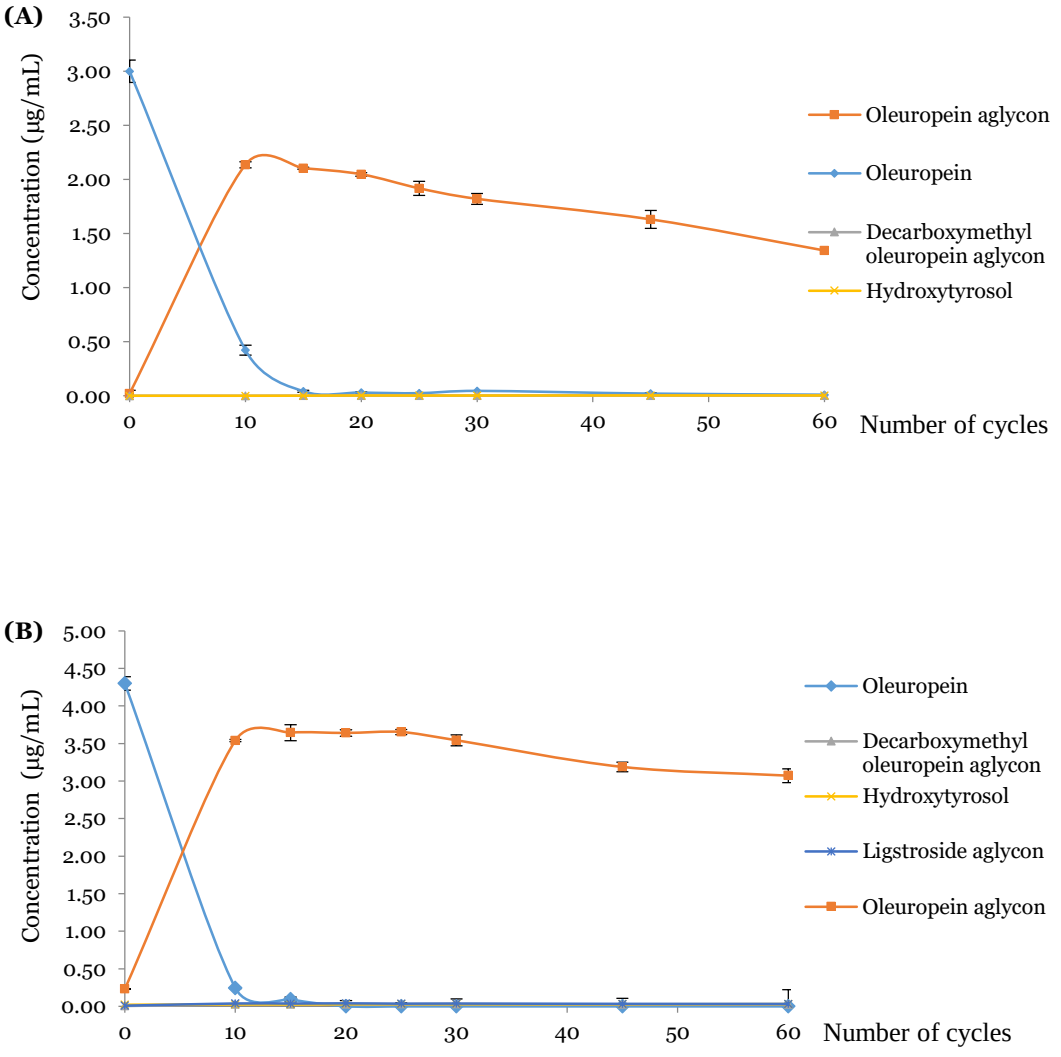

Fig. S4. USAEH (0–60 cycles) of oleuropein from an analytical standard (3.0 µg/mL) **(A)**, and from an olive leaf extract **(B)**.

Chapter V

Tentative identification of the composition of *Agaricus bisporus* aqueous enzymatic extracts with antiviral activity against HCV: A study by liquid chromatography–tandem mass spectrometry in high resolution mode

Tentative identification of the composition of *Agaricus bisporus* aqueous enzymatic extracts with antiviral activity against HCV: A study by liquid chromatography–tandem mass spectrometry in high resolution mode

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Tentative identification of the composition of *Agaricus bisporus* aqueous enzymatic extracts with antiviral activity against HCV: A study by liquid chromatography–tandem mass spectrometry in high resolution mode

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Abstract

Aqueous enzymatic extracts of edible mushroom *Agaricus bisporus* (AbAEE) have been studied as a key source of biological active compounds, among which those with antiviral activity against HCV are of special interest. Searching for a better knowledge of AbAEE, a new approach based on sequential fractionation with solvents of different polarity is here proposed to improve the tentative identification of AbAEE components by liquid chromatography–tandem mass spectrometry (LC–QTOF MS/MS) in high resolution mode. Fifty five out of one hundred twenty-three total metabolites tentatively identified by combination of MS and MS/MS information were identified for the first time. The identified compounds include amino acids, sugars, carboxylic, fatty and cinnamic acids, mono- and disaccharides, phospholipids and purines, as the most outstanding; thus demonstrating that fractionation based on sequential liquid–liquid extraction followed by LC–QTOF MS/MS is a suited option to obtain a wide, representative snapshot of AbAEE composition.

Keywords: mushroom, *Agaricus bisporus*, aqueous enzymatic extracts, liquid chromatography–tandem mass spectrometry, antiviral activity, HCV.

1. Introduction

Besides their unique flavor and sensory properties, mushrooms are endowed with excellent nutritional and healthy properties; therefore, mushrooms have been appreciated and consumed for thousands of years thanks to their nutritional value and health benefits. On a dry weight basis, mushrooms can be considered a good source of proteins, carbohydrates and dietary fiber (Barros, Baptista, Estevinho, & Ferreira, 2007), and of bioactive metabolites involved in health promotion and disease prevention (Bishop *et al.*, 2015). Currently, edible mushrooms and their extracts or fractions are considered good candidates to be included in healthy diets, as components of new functional foods or nutraceuticals. Concerning disease prevention, we recently described the "*in vitro*" inhibition of the protease NS3 of hepatitis-C virus (HCV) by aqueous enzymatic extracts of the common edible mushroom *Agaricus bisporus* (*A. bisporus*) (Gayego-Yerga & Bautista, 2015). This fact suggests incorporation of these extracts to the diet as components of new functional foods or nutraceuticals for hepatitis-C prevention in people under risk of HCV infection.

The use of these extracts as they are, as part of new functional foods or as nutraceuticals requires characterization of *A. bisporus* aqueous enzymatic extracts (AbAEE) as exhaustive as possible, in addition to complete safety. As far as we know, this characterization has not yet been carried out. In fact, the widest study on the chemical composition and biological properties of mushrooms and their extracts involved individual profiles of sugars (four sugars), fatty acids (twenty-three fatty acids), and tocopherols (three tocopherols) by using a gas chromatograph coupled to either a refractive index detector or a flame ionization detector (GC–FID), and by a liquid chromatograph coupled to a UV detector (LC–UV) (Barros, Venturini, Baptista, Estevinho, & Ferreira, 2008). Other compounds such as phenols, flavonoids, carotenoids or ascorbic acid have also been determined (Barros *et al.*, 2008); however, the study did not include determination of compounds such as peptides, organic acids or 5´-nucleotides. Triterpenes from *Ganoderma lucidum* have been determined by liquid chromatography–mass spectrometry (LC–QTOF MS/MS) and NMR (Dudhgaonkar, Thyagarajan, & Sliva, 2009). A different study was aimed at determining the EC_{50} value of extracts from *Boletus spp.* to evaluate their potential antioxidant activity, also including the targeted identification of potential antioxidant metabolites involved in the 1,1-diphenyl-2-picrylhydrazyl oxidation assay using

LC–QTOF MS/MS (Yuswan *et al.*, 2015). None of these studies on mushroom were targeted at overall identification.

Concerning *A. bisporus,* one of the major cultivated mushrooms*,* and its extracts the main studies have been addressed to the determination of the total content of moisture, protein, fat, carbohydrate and ash (Maseko *et al.*, 2013), carbohydrate composition; chemical characterization and speciation analysis of organic selenium compounds (Maseko *et al.*, 2013); determination of the antioxidant activity of phenols or polysaccharides (Bao, Ochiai, & Ohshima, 2010; Thetsrimuang, Khammuang, & Sarnthima, 2011); analysis of amino acids and 5´-nucleotides (Liu, Huang *et al.*, 2014), that of five organic acids by LC–UV or organic acids by LC–UV (Valentaõ *et al.*, 2005), that of no hallucinogenic indole compounds (Muszyńska, Sułkowska-Ziaja, Hałaszczuk, Krężałek, & Łojewsk, 2014), or the analysis of intact and purified lipid extracts from *A. bisporus* using 1D and 2D COSY proton NMR (Bonzom, Nicolaou, Zloh, Baldeo, & Gibbons, 1999). Other studies have involved identification of soluble sugars and polyols by LC equipped with an evaporative light scattering detector, free amino acids by LC–MS/MS, 5′-nucleotides by LC–UV at 254 nm, and organic acids by LC–UV at 210 nm (Pei *et al.*, 2014).

Therefore, the aim of the present study was the tentative identification of as many AbAEE components as possible by an appropriate sample preparation strategy for fractionation based on sequential liquid–liquid extraction (LLE) of AbAEE prior to LC–QTOFMS/MS in high resolution mode to obtain the required analytical information, increasing knowledge for a safe use of these extracts as they are, as part of new functional foods, or as nutraceuticals.

2. Materials and methods

2.1. Samples and chemicals

White button mushrooms (*A. bisporus*) were used as raw material after cultivation according to standard procedures in a pilot plant in the University of Seville (Spain). SensoLyte520 HCV Protease Assay Kit “Fluorimetric” and HCV NS3/4A protease were from *AnaSpec* (San Jose, CA, USA).

MS-grade ethyl acetate, dichloromethane and *n*-hexane from Sigma–Aldrich (Madrid, Spain) were used as extractants for LLE. MS-grade formic acid (FA) from Scharlab (Barcelona, Spain) was used as ionization agent. Methanol MS-grade from Sigma–Aldrich, and LC–grade from Scharlab were used to prepare the chromatographic mobile phases for LC–MS/MS and LC–diode array detector (DAD) analysis, respectively. Deionized water (18 mΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the aqueous solutions.

2.2. Extracts preparation

AbAEE was obtained by an enzymatic procedure based on that described by Cremades *et al.* (2012). Briefly, after homogenization and enzymatic digestion, the hydrolyzate was centrifuged at 8000×*g*, resulting in a supernatant and a solid residue. The latter (insoluble material) was discharged, and the supernatant (raw extract) was collected and filtrate through a Vivaflow-50 crossflow cassette (Sartorius), equipped with a 50 kDa ultrafiltration membrane, using the ultrafiltrate (UF-50) as AbAEE for activity assays.

2.3. Apparatus and instruments

A 5 liter reactor with pH, temperature and agitation control was used for enzymatic treatment, and a Vivaflow-50 crossflow cassette (Sartorius) was the ultrafiltration device. A Synergy HT plate reader (BioTeK) was use for enzymatic activity measurements. A vortex shaker from IKA (Wilmington, NC, USA), a Digtor 21 centrifuge from Ortoalresa (Madrid, Spain) and a Concentrator Plus speed-vac from Eppendorf (Hamburg, Germany) were used. An Agilent 1100 Series LC system from Agilent Technologies (Waldbronn, Germany) coupled to a DAD was used. Control of the instrumental setup and data acquisition was performed by the HPLC ChemStation software from Agilent.

An Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer (Santa Clara, CA, USA) equipped with dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution to calibrate continuously detected m/z ratios. The samples were thus monitored in high resolution mode. The Agilent MassHunter Workstation software was used to process the raw MS data,

including extraction of molecular features (MFs), generation of molecular formula, library searching and database searching.

2.4. HCV NS3-protease assay

HCV NS3-protease activity was fluorimetrically measured using the SensoLyte520 HCV Protease Assay Kit (Fluorimetric) and a recombinant HCV NS3/4A protease (*AnaSpec,* San Jose, CA, USA). 0.1 mg/mL of mushroom extracts (crude and AgAEE), together with 0.2 ng of freshly diluted enzyme were added to each well of a 96-well black assay plate (BD Falcon). The reaction started by adding 25 µL of freshly diluted substrate (1:25 in assay buffer solution). After incubation at room temperature (28 ºC) for 15 min, the fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ 490/520 nm in a Synergy HT, BioTeK plate reader. Substrate–enzyme reaction with vehicle dimethyl sulfoxide (DMSO) or water was used as reference value (100% activity). Inhibition percentages were calculated as:

$$\% \text{ inhibition} = 100\% - (RFU_o/RFU_x)\ 100\% \qquad (1)$$

where RFU_o and RFU_x are the fluorescence intensity of NS3 in the absence and presence of inhibitor, respectively. Quercetin (50 µM) and Telaprevir (TLP) (5µM) were used as positive inhibition controls. All analyses were performed in triplicate and the vehicle DMSO or water were considered as control (no inhibition, 100% of RFU).

2.5. Sequential fractionation of AbAEE

Fractionation of AbAEE was performed using sequential LLE with three extractants characterized by different polarity indices (pI), all of them immiscible with water, used in the following order: hexane (pI=0), dichloromethane (pI=3.1), and ethyl acetate (pI=4.4). For this purpose, 2 mL of AbAEE was diluted with 4 mL of deionized water and shaken for 1 min in the vortex before addition of 3 mL of hexane (first extractant) and shaken for 30 s in the vortex. The resulting two-phase system was centrifuged at 1500×*g* for 5 min for phase separation. The hexane phase was collected in a vial and the aqueous phase was shaken in the vortex with 3 mL of dichloromethane (second extractant) for 30 s.

The liquid–liquid system was centrifuged at 1500×*g* for 5 min and the dichloromethane phase was collected in a vial. Finally, 3 mL of ethyl acetate (third

extractant) was added to the remaining aqueous phase and the system shaken for 30 s in the vortex. The two-phase system was centrifuged at 1500×g for 5 min and each of the phases was collected in a vial. The extraction sequence is shown in Fig S1.

Then, 2 mL of each extract (hexane, dichloromethane and ethyl acetate extracts) was concentrated up to 0.5 mL in the speed-vac prior to analysis.

2.6. LC–DAD analysis

The analytical column was a 5 μm particle size, 250 mm×4.6 mm i.d. C18 Inertsil ODS-2 from GL Sciences Inc. (Torrance, CA, USA), and a C18 pre-column, 4 mm×3.0 mm i.d., from Phenomenex (Torrance, CA, USA) was used to protect the analytical column. The mobile phases were: 0.1% FA in water (phase A) and 0.1% FA in methanol (phase B). Three chromatographic gradients, at 0.8 mL/min constant flow rate, were used according to the polarity of the extracts. For no polar extracts (preconcentrated and no preconcentrated hexane extracts) the initial mobile phase was 96% A and 4% B, changed to 100% B along 30 min, then maintained constant for 30 min more. For medium polar extracts (preconcentrated and no preconcentrated both dichloromethane and ethyl acetate extracts) the initial mobile phase was 96% A and 4% B, changed to 100% B along 30 min, then maintained for 12 min more. For high-polar analytical samples (1:2 diluted remaining aqueous phase and 1:10 diluted MAEE, in both cases diluted with water) the initial mobile phase was also 96% A and 4% B, changed to 75% A and 25% B between min 0 and 30, to 100% B between min 30 and 40, then maintained for 2 min more.

In all cases a 10 min post-time was set to equilibrate the initial conditions for the next analysis. The injection volume was 20 μL and the injector needle was washed for 5 times between injections with 70% acetonitrile to avoid cross contamination. The chromatograms were acquired at 225, 250, 260, 280 and 320 nm (wavelengths of maximum absorption for most phenols).

2.7. LC–QTOF MS/MS analysis

Identification was carried out by LC–QTOFMS/MS analysis in high-resolution mode due to the complexity of mushroom extracts. The analytical samples were injected into the LC–QTOF system without additional preparation, except for the remaining

aqueous phase and the AbAEE, which were 1:10 and 1:30, respectively, diluted with water. The injection and separation conditions were identical to those for the LC–DAD analysis, except for the use of LC–MS grade solvents.

The parameters of the ESI source, operating in negative and positive ionization modes, were as follows: the capillary and fragmentor voltage were set at ± 3.5 kV and 145 V, respectively; N_2 in the nebulizer was flowed at 40 psi; the flow rate and temperature of the N_2 as drying gas were 10 L/min and 350 ºC, respectively. The instrument was calibrated and tuned according to the procedures recommended by the manufacturer. Data were collected in both centroid modes at a rate of 1 spectrum per second in the extended dynamic range mode (2 GHz). Accurate mass spectra in MS scan were acquired in the *m/z* range 60–1100 and in MS/MS mode in the *m/z* range 31–1100. The instrument gave typical resolution 15,000 FWHM (Full Width at Half Maximum) at *m/z* 118.0862 and 30,000 FWHM at *m/z* 922.0098. To assure the desired mass resolution, continuous internal calibration was performed during analyses by using the signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonatedhexakis(1H,1H,3H-tetrafluoro-propoxy)phosphazine or HP-921] in the positive ion mode; while in the negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 1033.9881 (TFA adduct of HP-921) were used.

The analytical samples were injected in auto MS/MS acquisition mode to obtain information from fragmentation of the target compounds. The maximum number of precursors selected per cycle was set at 2, with an exclusion window of 0.1 min after 2 consecutive selections of the same precursor. Collision energy of 20 eV was used to obtain the maximum information from fragmentation. Two replicates of each analytical sample were analyzed.

2.8. Data pretreatment

MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by LC–QTOF in full scan MS mode. Treatment of raw data files started by extraction of potential MFs with the suited algorithm included in the software. For this purpose, the extraction algorithm considered all ions exceeding 800 counts for negative ionization mode and 1000 counts for positive ionization mode with a single charge state for the obtained chromatograms. These

cut-off values were established taking into account the chromatographic background noise. Additionally, the isotopic distribution to consider MFs as valid should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/z*, plus 7.0 ppm in mass accuracy). Ions and adducts formation in the positive (+H, +Na) and negative ionization (−H, −2H, +HCOO, +Cl) modes, as well as neutral loss by dehydration, were included to identify features corresponding to the same potential metabolite. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention time (RT), intensity in the apex of the chromatographic peaks and accurate mass. In this way, raw data files were created in compound exchange format files (.cef files) for each sample and exported into the Mass Profiler Professional software package (version 13.0, Agilent Technologies, Santa Clara, CA, USA) for further processing.

In the next step, the data were processed by alignment of RT and *m/z* values across the data matrix using a tolerance window of 0.6 min and 10 ppm mass accuracy, respectively. Stepwise reduction of the MFs number was based on frequency of occurrence by comparing repetitions of the same sample.

Identification of the most relevant entities was supported on MS and MS/MS information and search in the METLIN MS and MS/MS databases (http://metlin.scripps.edu) and the Human Metabolome Database (HMDB, 3.6 version).

2.9. Statistical analysis

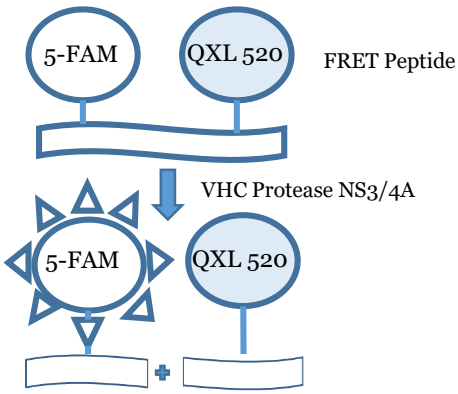
All inhibition experiments were conducted in triplicate. Statistical analysis was carried out using SPSS (version 16.0, Chicago, USA). The results were expressed as mean±standard deviation, and the significance was set at 95% confidence level.

3. Results and discussion

3.1. HCV protease NS3/NS4 inhibition

In vitro inhibitory capacity of crude extract and AbAEE against the HCV recombinant protease NS3/NS4 was studied by a fluorimetric assay using the SensoLyte®

520 HCV Protease Assay Kit. This procedure provides an appropriate assay for high throughput screening of HCV NS3/4A protease inhibitors and for continuous quantitation of HCV NS3/4A protease activity using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. The sequence of this peptide is derived from the cleavage site of NS4A/NS4B. In the FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by HCV NS3/4A protease (Scheme 1), the recovered fluorescence of 5-FAM can be monitored at $\lambda_{ex}/\lambda_{em}$ = 490/520 nm. Thanks to a higher fluorescence quantum yield and longer wavelength, the signal of 5-FAM is less interfered by the auto fluorescence of cell components and test compounds. The assay can detect as low as 1.56 ng/mL active HCV NS3/4A protease (Tong *et al.*, 2005).



Scheme 1. Proteolytic cleavage of 5-FAM/QXL™520 FRET peptide by HCV NS3/4A protease.

Fig. 1 shows that VHC protease NS3/NS4 activity is 64.85% and 75.56% inhibited by *A. bisporus* raw extract (41.15 µg/mL) and AgAEE (20.73 µg/mL), respectively; while quercetin (50 µM = 15.122 µg/mL), and telaprevil (TPV, 5 µM = 3.40 µg/mL), a natural and a synthetic inhibitors of the VCH NS3 protease that inhibit 29.86 and 87.69%, respectively, the recombinant enzyme. These data show the significant inhibition of VCH protease by AgAEE; inhibition greater than that of quercetin 50 µM; but lower than that caused by the synthetic inhibitor TPV. However, it must be taken into account that while quercetin and TPV are pure products, AgAEE are complex mixtures of at least 123 different compounds (Table 1), as discussed below. Therefore, a significant increase of the inhibitory activity can be expected after extracts purification. Consequently, isolation and identification of the product or products responsible for the inhibition is of utmost importance.

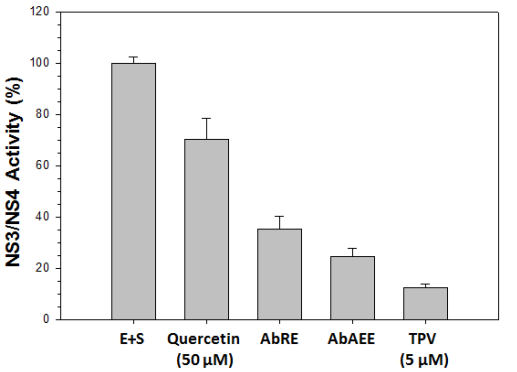


Fig. 1. *In vitro* inhibition of recombinant protease NS3/NS4 of HCV by *A. bisporus* raw extract (AbRE), *A. bisporus* aqueous enzymatic extract (AbAEE), quercetin and telaprevir (TPV).

Table 1. List of metabolites identified in the different fractions and AbAEE classified by families. RT is included, as well as the MS identification information (precursor and product ions) and error (expressed as ppm) of the precursor ion detected. In bold are metabolites identified for the first time in this research.

Metabolite	MS spectra experimental (*m/z*)	MS spectra theoretical (*m/z*)	Error (ppm)	Adduct	RT (min)	Product ions (*m/z*)	Extract
Alkaloid derivative							
2-Methyl-pyrrolidine	86.0969	86.0970	1.2	$[M+H]^+$	7.90	43.0539 41.0390	AbAEE[1], Aq[2]
Alkylamines							
Pantothenic acid	220.1178	220.1186	3.6	$[M+H]^+$	20.02	90.0556 72.0443	AbAEE, Aq
Spermidine	146.1651	146.1658	4.8	$[M+H]^+$	2.53	84.0811 72.0809	AbEE, Aq
Pyrroline							
1-Pyrroline	70.0652	70.0657	7.1	$[M+H]^+$	4.28	43.0542 41.0385	AbAEE, Aq
Amino acids and derivatives							
2,5-Dihydrophenyl-alanine	168.1019	168.1025	3.6	[M+H]+	6.78	126.0551 81.0707	Dclo cc[3], Dclo[4], Et[5], Et cc[6]
2-Amino-butyric acid	102.0562	102.0554	−7.8	[M−H]−	3.92	102.0562	AbAEE, Aq
3-Amino-2-naphthoic acid	188.0703	188.0712	4.8	[M+H]+	22.23	170.0594 142.0651	AbAEE, Aq
3-Amino-3-(4-hydroxy-phenyl)-propanoate	180.0665	180.0660	−2.8	[M−H]−	27.28	119.0495 93.0340	AbAEE, Aq

Continuation Table 1

5-Amino pentanoate	118.0864	118.0869	4.2	[M+H]+	4.20	74.0588 58.0625	AbAEE, Aq
Acetyl-carnitine	204.1231	204.1237	2.9	[M+H]+	4.04	85.0288 60.0809	AbAEE, Aq
Citrulline	176.1031	176.1036	2.8	[M+H]+	3.92	113.0708 70.0650	AbAEE, Aq
Cyclo-leucine	130.0863	130.0869	4.6	[M+H]+	5.52	84.0809 67.0540	AbAEE, Aq
Cystathionine	223.0746	223.0753	3.1	[M+H]+	3.55	134.0267 88.0212	AbAEE, Aq
Ergothioneine	230.0957	230.0964	3.0	[M+H]+	5.13	127.0322 60.0809	AbAEE, Aq
L-2-Amino-adipic acid	160.0616	160.0609	−4.4	[M−H]−	4.21	116.0720 98.0609	AbAEE, Aq
L-Agaritine	268.1292	268.1298	2.2	[M+H]+	7.31	84.0439 77.0389	AbAEE, Aq
L-Arginine	173.1040	173.1038	−1.1	[M−H]−	3.44	131.0823 112.0868	Aq
L-Asparagine	133.0610	133.0614	3.0	[M+H]+	3.70	74.0233 46.0283	AbAEE, Aq
L-Aspartic acid	132.0302	132.0296	−4.5	[M−H]−	4.02	88.0405 71.0139	AbAEE, Aq
L-Carnitine	162.1124	162.1131	4.3	[M+H]+	3.52	103.0389 60.0808	AbAEE, Aq
L-Glutamate	148.0602	148.0611	6.1	[M+H]+	3.94	85.0284 56.0494	AbAEE, Aq
L-Glutamine	147.0761	147.0770	6.1	[M+H]+	3.77	84.0442 56.0493	AbAEE, Aq
L-Isoleucine	132.1019	132.1025	4.5	[M+H]+	7.40	86.0967 69.0699	AbAEE, Aq
LL-2,6-Di-aminohep-tanedioate	189.0886	189.0875	−5.8	[M−H]−	4.16	84.0444 74.0237	AbAEE, Aq
L-Leucine	132.1020	132.1025	3.8	[M+H]+	7.87	86.0967 44.0492	AbAEE, Aq
L-Lysine	147.1129	147.1134	3.4	[M+H]+	2.77	84.0809 56.0492	Aq
L-Methionine	150.0585	150.0590	3.3	[M+H]+	5.80	61.0105 56.0496	AbAEE, Aq
L-Phenyl-alanine	166.0860	166.0869	5.4	[M+H]+	14.21	120.0804 103.0544	AbAEE, Aq
L-Proline	116.0703	116.0712	7.8	[M+H]+	4.28	70.0652 43.0539	AbAEE, Aq
L-Serine	104.0353	104.0347	−5.8	[M−H]−	3.75	74.0243 42.0356	AbAEE, Aq
L-Threonine	120.0651	120.0661	8.3	[M+H]+	3.79	74.0599 56.0495	AbAEE, Aq
L-Tryptophan	205.0972	205.0978	2.9	[M+H]+	22.30	146.0600 118.0649	AbAEE, Aq
L-Tyrosine	182.0806	182.0818	6.6	[M+H]+	8.93	136.0753 91.0542	AbAEE, Aq
L-Valine	118.0863	118.0869	5.1	[M+H]+	4.81	72.0809 55.0543	AbAEE, Aq
N2-Acetyl-L-ornithine	175.1078	175.1083	2.9	[M+H]+	4.57	116.0692 70.0653	AbAEE, Aq
N6-Acetyl-L-2,6-di-aminohep-tanedioate	233.1134	233.1138	1.7	[M+H]+	6.54	87.0433 84.0465	AbAEE, Aq

Continuation Table 1

N-Carbamylglutamate	189.0518	189.0511	−3.7	[M−H]−	7.46	128.0347 102.0560	AbAEE, Aq
Nε-Acetyl-L-lysine	189.1230	189.1240	5.3	[M+H]+	5.29	126.0909 84.0811	AbAEE, Aq
Ornithine	133.0973	133.0978	3.8	[M+H]+	2.77	116.0708 70.0651	Aq
Pipecolic acid	130.0864	130.0869	3.8	$[M+H]^+$	5.15	84.0810 56.0493	AbAEE, Aq
Pyroglutamic acid	128.0353	128.0347	−4.7	$[M-H]^-$	9.09	128.0352 82.0294	AbAEE, Aq
Saccharopine	277.1393	277.1400	2.5	$[M+H]^+$	3.81	130.0864 84.0809	AbAEE, Aq
Anthranilic acid	138.0546	138.0556	7.2	$[M+H]^+$	12.04	120.0441 92.0500	Et cc
Peptides							
Cyclo(L-phe-L-pro)	245.1286	245.1291	2.0	$[M+H]^+$	19.78	120.0802 70.0654	Dclo, Dclo cc
Oxidized glutathione	611.1446	611.1441	−0.8	$[M-H]^-$	9.52	306.0762 272.0886	AbAEE, Aq
γ-D-Glutamylglycine	205.0822	205.0825	1.5	$[M+H]^+$	4.39	142.0494 84.0442	AbAEE, Aq
Quinoline derivative							
5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-tetrahydroquinoline	194.0812	194.0818	3.1	$[M+H]^+$	11.41	120.0455 57.0332	Et, Et cc
Indoles							
Formyl indole	146.0596	146.0607	7.5	$[M+H]^+$	20.48	118.0649 91.0543	Dclo, Dclo cc, Et cc
5-Hydroxyindolepyruvate	254.0224	254.0226	0.8	$[M+Cl]^-$	9.51	200.0353 191.0350	Et cc
Indole-3-acetate	174.0559	174.0554	−2.9	$[M-H]^-$	18.88	144.0439 130.0656	Dclo, Dclo cc
Indole-3-carboxylic acid	160.0403	160.0398	−3.1	$[M-H]^-$	20.40	116.0502	Et cc
Benzaldehyde							
4-Hydroxybenzaldehyde	123.0444	123.0447	2.4	$[M+H]^+$	8.92	95.0484 77.0381	AbAEE, Dclo cc, Aq
Benzoic acid derivative							
Salicylic acid	137.0242	137.0238	−2.9	$[M-H]^-$	17.07	93.0342 65.0396	Et cc
Carboxylic acids and derivatives							
2,2-Dimethyl succinic acid	145.0505	145.0500	−3.4	$[M-H]^-$	25.65	101.0602 83.0504	AbAEE, Aq
3-Hydroxy-3-methylglutaric acid	161.0454	161.0449	−3.1	$[M-H]^-$	11.98	59.0143 57.0349	AbAEE, Aq

Continuation Table 1

Citric acid	191.0195	191.0191	−2.1	$[M-H]^-$	9.73	111.0093 87.0089	AbAEE, Aq
Lactic acid	89.0246	89.0238	−9.0	$[M-H]^-$	6.98	43.0188 41.0032	AbAEE, Aq
Malic acid	133.0142	133.0136	−4.5	$[M-H]^-$	6.23	72.9928 71.0135	AbAEE. Aq
Succinic acid	117.0198	117.0187	−9.4	$[M-H]^-$	10.19	73.0297 55.0194	Aq
Cinnamic acid derivative							
p-Coumaric acid	165.0542	165.0552	6.1	$[M+H]^+$	19.57	147.0442 119.0493	AbAEE, Et cc, Aq
Disaccharides							
Cellobionic acid	357.1043	357.1032	−3.1	$[M-H]^-$	5.36	113.0233 89.0233	AbAEE, Aq
α-1,5-L-Arabino-biose	281.0876	281.0872	−1.4	$[M-H]^-$	8.44	89.0244 71.0132	AbAEE, Aq
Monosaccharides							
Sedohep-tulose-7-phosphate	289.0332	289.0324	−2.8	$[M-H]^-$	6.33	96.9693 78.9592	AbAEE, Aq
D-Glucosa-mine 6-phosphate	258.0385	258.0378	−2.7	$[M-H]^-$	5.92	171.0060 78.9615	Aq
D-Glucose 6-phos-phate	259.0226	259.0218	−3.1	$[M-H]^-$	6.34	96.9693 78.9592	AbAEE, Aq
D-Mannitol	183.0862	183.0869	3.8	$[M+H]^+$	4.12	69.0337 57.0330	AbAEE, Et, Aq
D-Mannitol 1-phosphate	261.0379	261.0375	−1.5	$[M-H]^-$	6.76	96.9694 78.9591	AbAEE, Aq
Sedoheptu-lose	209.0664	209.0661	−1.4	$[M-H]^-$	4.83	59.0128 57.0335	AbAEE, Aq
α-D-Glucose	179.0556	179.0555	−0.6	$[M-H]^-$	4.62	71.0141 59.0134	AbAEE, Aq
Sugar acids and derivatives							
Galactonic acid	195.0511	195.0504	−3.6	$[M-H]^-$	4.32	75.0090 59.0139	AbAEE, Aq
Glucuronic acid	193.0349	193.0348	−0.5	$[M-H]^-$	4.56	75.0088 59.0139	AbAEE, Aq
Glyceric acid	105.0189	105.0187	−1.9	$[M-H]^-$	4.90	59.0143 56.9850	AbAEE, Aq
2-Dehydro-3-deoxy-L-arabinonate	147.0300	147.0293	−4.8	$[M-H]-$	8.06	85.0284 57.0335	AbAEE, Aq
Glycerol-2-phosphate	171.0066	171.0058	−4.7	$[M-H]-$	6.68	96.9688 78.9594	AbAEE, Aq
Xylonate	165.0402	165.0398	−2.4	$[M-H]-$	4.48	75.0089 59.0140	AbAEE, Aq
Fatty acids and conjugates							
2-Hydroxy-palmitic acid	271.2282	271.2272	−3.7	$[M-H]-$	29.23	253.2157 225.2225	Hex cc7, Dclo, Dclo cc
2-Hydroxy-isocaproic acid	131.0715	131.0707	−6.1	$[M-H]-$	38.15	85.0660 69.0340	AbAEE, Aq

Continuation Table 1

3-Hydroxy-palmitic acid	271.2281	271.2272	−3.3	$[M-H]^-$	31.46	59.0162	Dclo cc
3-Hydroxy-myristic acid	243.1969	243.1959	−4.1	$[M-H]^-$	29.97	243.1950 59.0142	Dclo cc
3-Hydroxy-capric acid	187.1341	187.1333	−4.3	$[M-H]^-$	26.38	59.0138 41.0031	Dclo cc, Et, Et cc
5,8-DiHODE	311.2229	311.2222	−2.2	$[M-H]^-$	26.52	167.1441 127.0765	Dclo cc, Et cc
6,7-Epoxy-stearic acid	297.2438	297.2429	−3.0	$[M-H]^-$	29.67	297.2427 185.1178	Hex[8], Hex cc, Dclo, Dclo cc
9,10,13-TriHOME	329.2339	329.2327	−3.6	$[M-H]^-$	25.40	157.1223 99.0804	Et cc
9,10-Dihydroxy-stearic acid	315.2541	315.2534	−2.2	$[M-H]^-$	27.89	298.2502 44.9971	Dclo, Dclo cc, Et cc
9,10-Epoxy-18-hydroxy-stereate	313.2382	313.2378	−1.3	$[M-H]^-$	27.32	113.0961 99.0804	Dclo, Dclo cc, Et cc
Oleic acid	281.2484	281.2480	−1.4	$[M-H]^-$	34.20	281.2501	Hex cc
ε-Capra-mine	132.1020	132.1025	3.8	$[M+H]^+$	4.54	59.0724 132.1018	AbAEE, Aq
Palmitic acid	255.2331	255.2323	−3.1	$[M-H]^-$	34.10	255.2337	Hex cc, Et cc
Palmitoleic acid	253.2170	253.2167	−1.2	$[M-H]^-$	32.56	253.2157	Hex cc
Stearic acid	283.2646	283.2636	−3.5	$[M-H]^-$	36.24	283.2638	Hex, Hex cc, Dclo, Dclo cc, Et
***α*-Licanic acid**	293.2089	293.2117	9.5	$[M+H]^+$	29.24	116.0505 45.0336	Hex cc, Dclo, Dclo cc
Fatty amides							
13-Docosena-mide	338.3418	338.3424	1.8	$[M+H]^+$	36.32	83.0855 57.0697	Hex, Hex cc, Dclo, Dclo cc, Et, Et cc
Oleamide	282.2793	282.2798	1.8	$[M+H]^+$	32.73	71.0861 57.0693	Hex cc, Dclo, Dclo cc, Et
Sphingolipids							
C16 Sphinganine	274.2739	274.2747	2.9	$[M+H]^+$	22.69	256.2622 106.0853	Dclo, Dclo cc
Sphinganine	302.3058	302.3060	0.7	$[M+H]^+$	24.34	284.2943 57.0692	Dclo, Dclo cc
Phospholipid and derivative							
Phospho-choline	184.0731	184.0739	4.3	$[M+H]^+$	4.04	124.9997 86.0967	AbAEE, Aq
Glycer ophospho-choline	258.1099	258.1107	3.1	$[M+H]^+$	4.14	124.9996 104.1070	AbAEE, Aq
Furan							
2-Furoic acid	111.0088	111.0081	−6.3	$[M-H]^-$	9.73	67.0189 39.0241	AbAEE, Aq
No metal oxoanionic compounds							
Phosphate	96.9694	96.9690	−4.1	$[M-H]^-$	5.80	96.9690 78.9594	AbAEE, Aq

Continuation Table 1

Phosphite	78.9588	78.9584	−5.1	$[M-2H]^-$	6.00	78.9599 62.9646	AbAEE, Aq
Phenol derivative							
p-Amino-phenol	110.0601	110.0607	5.5	$[M+H]^+$	3.37	93.0336 65.0387	Dclo, Dclo cc, Et, Et cc, Aq
Purine nucleoside derivative							
Xanthosine	283.0650	283.0678	9.9	$[M-H]^-$	18.19	151.0260	AbAEE, Aq
Purine nucleotides and derivatives							
3´-AMP	346.0559	346.0552	−2.0	$[M-H]^-$	13.50	211.0014 78.9590	AbAEE, Aq
AMP	348.0702	348.0710	2.3	$[M+H]^+$	8.83	136.0619 97.0285	AbAEE, Aq
ADP	426.0220	426.0215	−1.2	$[M-H]^-$	13.01	158.9250 78.9593	AbAEE, Aq
cGMP	344.0405	344.0395	−2.9	$[M-H]^-$	21.66	150.0421 108.0203	AbAEE, Aq
GDP	442.0173	442.0164	−2.0	$[M-H]^-$	17.73	344.0402 150.0421	AbAEE, Aq
GDP-mannose	604.0701	604.0693	−1.3	$[M-H]^-$	15.48	442.0177 424.0057	AbAEE, Aq
GMP	364.0654	364.0659	1.4	$[M+H]^+$	12.53	152.0567 97.0289	AbAEE, Aq
N6-(1,2-Dicarboxy-ethyl)-AMP	464.0814	464.0819	1.1	$[M+H]^+$	28.45	252.0724 234.0620	AbAEE, Aq
Purines and purine derivatives							
Adenine	134.0469	134.0466	−2.2	$[M-H]^-$	4.78	107.0360 92.0248	AbAEE, Dclo, Dclo cc, Et, Et cc, Aq
Guanine	152.0568	152.0573	3.3	$[M+H]^+$	5.34	135.0302 110.0351	AbAEE, Aq, Et
Hypoxanthine	137.0454	137.0464	7.3	$[M+H]^+$	9.31	119.0351 110.0349	AbAEE, Aq, Et, Et cc
Uric acid	167.0210	167.0204	−3.6	$[M-H]^-$	9.22	124.0154 41.9985	AbAEE, Aq
Xanthine	151.0260	151.0255	−3.3	$[M-H]^-$	11.07	108.0196 41.9987	AbAEE, Aq, Et, Et cc
Pyrimidine nucleotides and derivatives							
UMP	323.0291	323.0280	−3.4	$[M-H]^-$	12.30	96.9691 78.9592	AbAEE, Aq
cUMP	305.0183	305.0174	−3.0	$[M-H]-$	15.51	111.0197 41.9985	AbAEE, Aq
UDP-D-xylose	535.0374	535.0366	−1.5	$[M-H]-$	19.31	323.0286 78.9591	AbAEE, Aq
UDP-glucose	565.0479	565.0471	−1.4	$[M-H]-$	18.90	384.9842 323.0289	AbAEE, Aq
UDP-glucuronic acid	579.0271	579.0264	−1.2	$[M-H]-$	19.54	402.9954 323.0289	AbAEE, Aq
UDP-N-acetylglucosamine	606.0744	606.0737	−1.2	$[M-H]-$	19.97	384.9850 282.0392	AbAEE, Aq

Continuation Table 1

Pyrimidine derivative							
Uracil	111.0199	111.0194	−4.5	$[M-H]^-$	7.01	41.9988	AbAEE, Et, Et cc
Pyridine derivative							
Nicotinic acid	124.0394	124.0399	4.0	$[M+H]^+$	7.09	80.0497 78.0335	AbAEE, Aq

AbAEE, *Agaricus bisporus* aqueous enzymatic extract; Aq, remaining aqueous extract; Dclo cc, preconcentrated dichloromethane extract; Dclo, dichloromethane extract; Et, ethyl acetate extract; Et cc, preconcentrated ethyl acetate extract; Hex cc, preconcentrated hexane extract; Hex, hexane extract.

3.2. Sequential fractionation of AbAEE

AbAEE are complex mixtures that mainly contain polar compounds due to their aqueous character (Kalač, 2009), but also no polar compounds are present in it. This complex composition justifies the selection of extractants with different polarity for sequential isolation of compounds pertaining to different families in order to enhance the coverage of identified compounds. The fractionation efficiency was evaluated by LC–DAD. The same mobile phases with slight modifications of the chromatographic gradients were used in all instances to facilitate comparison. Fig. 2 shows the DAD chromatograms provided by the analysis of the different fractions. Thus, Fig. 2A corresponds to the hexane extract (chromatogram obtained at 280 nm), Fig. 2B to the dichloromethane extract (at 260 nm), Fig. 2C to the ethyl acetate extract (at 260 nm), Fig. 2D to the remaining aqueous phase (at 260 nm), and Fig. 2E to the analysis of MAEE (at 260 nm). Comparison of the chromatograms A-to-E in Fig. 2 emphasizes: (i) the complementarity of the fractionation, as shows the retention time distribution of the peaks in the different chromatograms; (ii) the polar character of most of the metabolites, shown by the high number of peaks found in the aqueous fraction; and (iii) the high concentration of polar compounds, taking into account that the analysis of AbAEE and the aqueous remaining fraction were 1:10 and 1:2 diluted, respectively; while the organic phases were four times concentrated prior to injection into the chromatograph.

Tentative identification was only applied to compounds with high quality MS/MS spectra. This step was supported on mass accuracy for precursor ions and also for the most representative product ions by searching for them in the METLIN database. Most of the compounds were detected in both positive and negative ionization polarities, but only the mode reporting the best resolution was included. A mass accuracy error for the precursor

ions lower than 10 ppm was accepted. The analysis of all analytical samples by LC–QTOF MS/MS allowed tentative identification of 123 compounds, while analysis of AbAEE without fractionation reported 90 compounds.

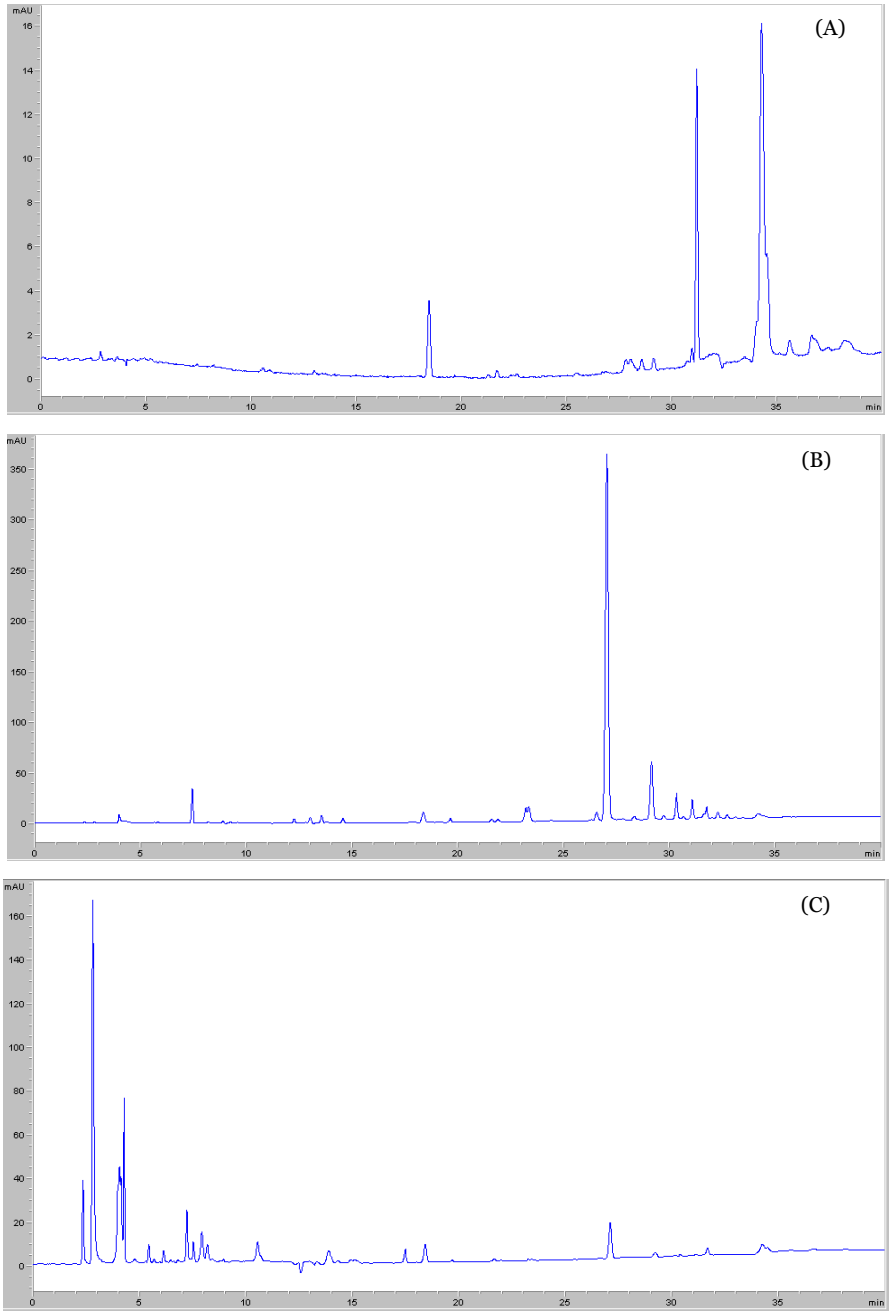


Fig. 2. LC–DAD chromatograms from the fractions of the **(A)** hexane extract (obtained at 280 nm); **(B)** dichloromethane extract (at 260 nm); and **(C)** ethyl acetate extract (at 260 nm).

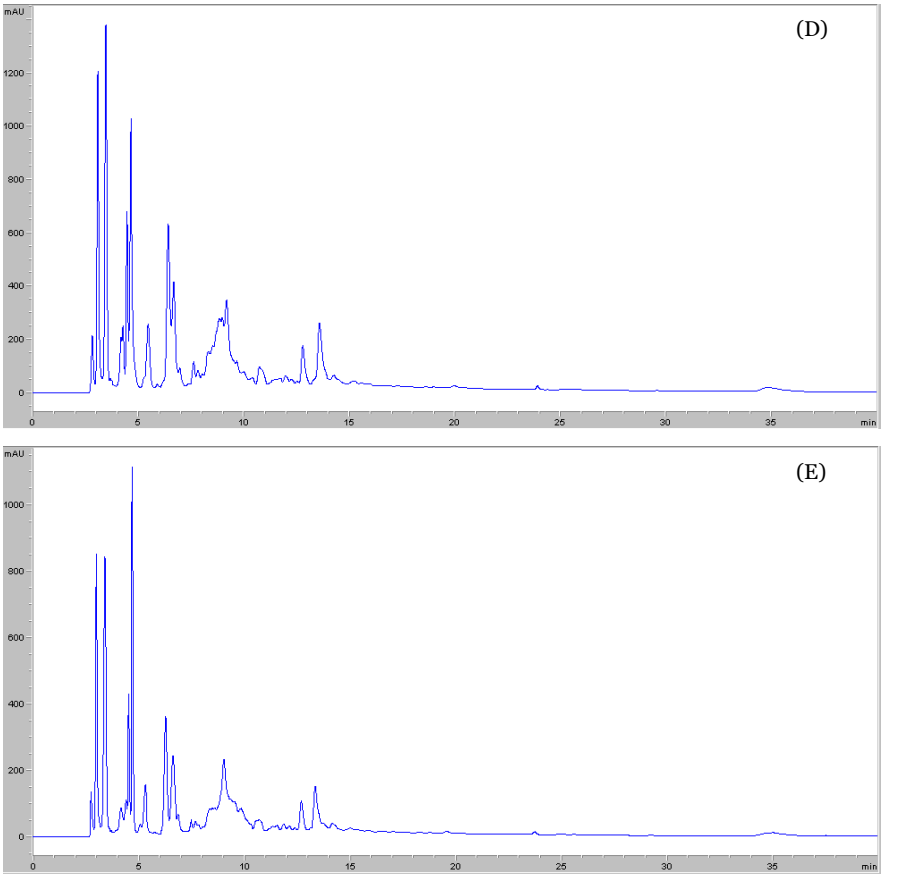


Fig. 2. LC–DAD chromatograms from the fraction of the **(D)** 1:2 diluted remaining aqueous phase (at 260 nm); and **(E)** that from the 1:10 diluted *A. bisporus* aqueous enzymatic extract (at 260 nm).

This identification increase can be attributed to fractionation as it permits implementing dilution or preconcentration steps for the different fractions as required.

Fig. 3 shows a Venn diagram in which all compounds identified in the four fractions (three organic phases plus the remaining aqueous phase) are compared with those identified in AbAEE. As can be seen, 90 compounds were common to all the samples, while 33 compounds were only identified in some of the fractions and no in the AbAEE. The compounds only identified in the fractionated extracts were mainly no polar (14 fatty acids and derivatives, 2 fatty amides and 2 sphingolipids), but also 5 amino acids, 4 indols, 1 peptide, 1 phenol derivative, 1 benzoic acid derivative, 1 carboxylic acid, 1 monosaccharide derivative and 1 quinoline derivative. Among the total 123 tentatively identified

compounds, 55 were identified for the first time in this fungus (43 in AbAEE and aqueous remaining phase and 12 in the organic fractions).

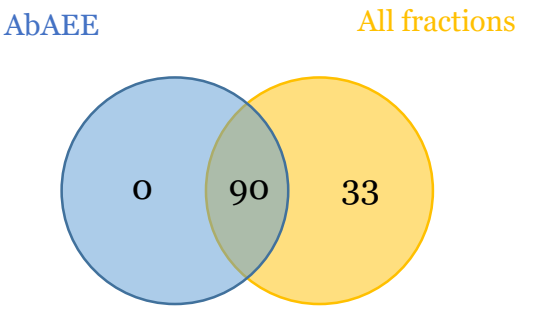


Fig. 3. Venn diagram comparing the compounds identified in *A. bisporus* aqueous enzymatic extract (AbAEE) with the sum of those identified in the four fractions.

Once the tentative identification was complete, the efficiency of fractionation was explained by Venn diagrams. Fig. 4 shows the Venn diagram involving the compounds identified in the analyzed fractions.

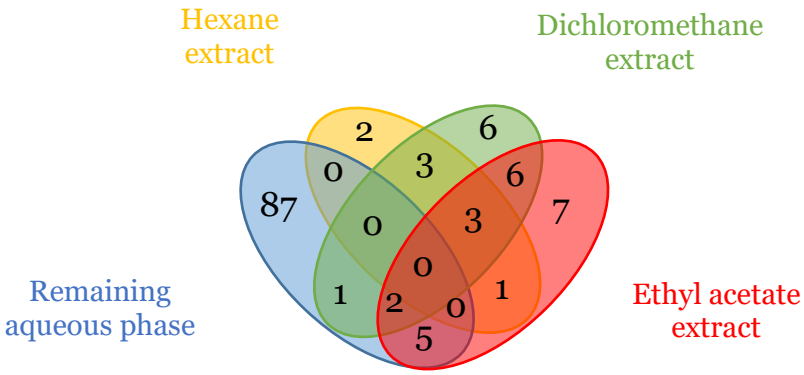


Fig. 4. Venn diagram comparing the compounds identified in the hexane, dichloromethane, ethyl acetate extracts and those remaining in the aqueous phase.

As can be seen, the number of compounds identified in the aqueous sample was clearly higher than in hexane, dichloromethane or ethyl acetate extracts. Thus, the analysis of the hexane extract allowed identification of 9 compounds, that of dichloromethane 21

compounds and that of ethyl acetate 24 compounds. The analysis of the remaining aqueous fraction allowed identifying 95 compounds. One fact to be emphasized is that some of the identified compounds were found in all analytical fractions, but most of them were exclusively identified in one of the fractions, thus confirming the complementarity achieved by the fractionation step.

3.3. Comparison of the metabolites tentatively identified in the no polar fraction (hexane extract) and AbAEE

The analysis of the hexane extract allowed identification of 9 compounds that included 4 fatty acids —palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), and oleic acids (C18:1)— and 5 fatty acid derivatives —2-hydroxypalmitic acid, 6,7-epoxystearic acid, α-licanic acid, oleamide and 13-docosenamide). None of them were among the 90 compounds tentatively identified in the direct analysis of AbAEE (see Fig. 5A), which is explained by the 1:30 dilution of AbAEE and the 4:1 preconcentration of the hexane extract applied to fit the scale of the analytical response.

The compounds tentatively identified in the hexane extract were fatty acids and conjugates, which represent an important source of energy and facilitate vitamins absorption. These compounds were previously described by Woldegiorgis *et al.* (Woldegiorgis, Abate, Haki, Ziegler, & Harvatine, 2015), who determined fatty acids in mushrooms by GC–FID, after methylation. Two main saturated fatty acids —*viz.,* palmitic (C16:0) and stearic (C18:0)— were tentatively identified in hexane extracts, in agreement with the literature that reports C16:0 as one of the most abundant fatty acids in *A. bisporus* (Nasiri, Ghiassi Tarzi, Bassiri, Hoseini, & Aminafshar, 2013). Unsaturated fatty acids such as oleic (C18:1) and palmitoleic (C16:1) acids, with modulatory effects on inflammatory diseases, have also been found in the hexane extract of the target mushroom, in agreement with previous studies on *Agaricus* species (Nasiri *et al.*, 2013), as did oleic acid, detected as amide derivative, in agreement with previous studies on mushroom (Hyun, Lee, Sung, Kim, & Choi, 2013). Other fatty amide tentatively identified in the hexane extract was 13-docosenamide, previously identified in mushroom by Nosaka *et al.* (Nosaka & Miyazawa, 2014). Other fatty acid derivatives detected in hexane extracts were the acids 2-hydroxypalmitic, α-licanic and 6,7-epoxystearic, among which 2-hydroxypalmitic acid had previously been identified (Prošteník *et al.*, 1978), while α-licanic acid and 6,7-epoxystearic

acid had not previously been identified in any mushroom specie —the former had been identified in flowering plants (*e.g.*, in seeds of *Chrysobalanaceae*) (Prance, 2014).

3.4. Comparison of the metabolites tentatively identified in the dichloromethane extract with those in either AbAEE or hexane extract

The analysis of the dichloromethane extract allowed tentative identification of 21 compounds *versus* 90 identified in AbAEE. Only 2 compounds (*viz.,* adenine and 4-hydroxybenzaldehyde) were common to both extracts; therefore, 19 compounds were exclusively identified in the dichloromethane extract and 88 in AbAEE. Ten fatty acids and derivatives such as hydroxy fatty acids and epoxy fatty acids, 2 fatty amides (oleamide and 13-docosenamide), 2 sphinganines (C16 sphinganine and sphinganine), 2 indoles (indole-3-acetate and formyl indole), 1 peptide, 1 amino acid, and 1 phenol were the compounds tentatively identified in the dichloromethane extract. Fig. 5B shows the Venn diagram that compares the detection coverage by analyses of AbAEE and dichloromethane extract.

Only 6 out of the 21 compounds tentatively identified in the dichloromethane extract were also identified in the hexane extract. Four fatty acids and derivatives (stearic acid, α-licanic acid, 6,7-epoxystearic acid and 2-hydroxypalmitic acid), and 2 fatty amides (oleamide and 13-docosenamide) were the compounds common to both extracts. Among the 15 compounds identified in the dichloromethane extract, but no in the hexane extract, 6 were fatty acids and derivatives, 2 sphinganines, 2 indoles, 1 peptide, 1 amino acid, 1 phenol, 1 purine and 1 benzaldehyde. Hydroxy fatty acids are useful as starting materials for functional foods (Takeuchi *et al.*, 2013); 3 of the identified hydroxy fatty acids were 3-hydroxy fatty acids (3-hydroxypalmitic acid, 3-hydroxymyristic acid and 3-hydroxycapric acid), which are typical of *Pseudomonads* (Saddler, 1994). Other 3 of the hydroxy fatty acids identified in dichloromethane extracts (5,8-DiHODE, 9,10-dihydroxystearic acid and 9,10-epoxy-18-hydroxystereate, 5,8-DiHODE and 9,10-dihydroxystearic acid) had previously been identified in mushroom (Kang *et al.*, 2013; Morris, 1968); while the 9,10-epoxy-18-hydroxystearate had not previously been identified in mushroom, but it did in barley (Chamarthi *et al.*, 2014). Two sphingolipids (*viz.,* sphinganine and C16 sphinganine) were identified in dichloromethane extracts, in agreement with the literature that reports up to 10 glucosylceramides in Maitake mushrooms (Sugawara, Aida, Duan, & Hirata, 2010). Dietary sphingolipids have gained attention because their potential to protect

intestine from inflammation (Duan & Nilsson, 2009). Other metabolites identified in dichloromethane extracts were indoles (formyl indole and indole-3-acetate) (Arnold, Palfner, Schmidt, Kuhnt, & Becerra, 2012; Muszyńska, Sułkowska-Ziaja, & Ekiert, 2013a). No essential compounds such as adenine, cyclo(L-phe-L-pro), 2,5-dihydrophenylalanine, 4-hydroxybenzaldehydeand *p*-aminophenol were also identified in dichloromethane extracts. Adenine, *p*-aminophenol (Weijn *et al.*, 2013), and 4-hydroxybenzaldehyde had previously been identified in mushroom (Papaspyridi *et al.*, 2012). *p*-Aminophenol is, together with other phenols, associated to browning, thus producing the most common postharvest changes in button mushrooms, which often result in economic losses (Weijn *et al.*, 2013). 4-Hydroxybenzaldehyde is a phytotoxic substance with allelopathic activity found in endophytic fungi *Aspergillus* sp. (Ren-Yi *et al.*, 2015); cycle(L-Phe-L-Pro) is one of the compounds with higher involvement in the antifungal activity of *Lactobacillus plantarum* FST 1.7 (Dalbello *et al.*, 2007), isolated from the *Pseudomonas aeruginosa* PAO1 strain; finally, 2,5-dihydrophenylalanine is a multipotent no proteinogenic amino acid produced by various actinobacterias and gammaproteobacterias (Crawford, Mahlstedt, Malcolmson, Clardy, & Walsh, 2011), but never identified in mushroom.

3.5. Comparison of the metabolites tentatively identified in ethyl acetate extract with those in AbAEE and the other less polar extracts

The analysis of the ethyl acetate extract allowed identification of 24 compounds *versus* the 90 compounds in AbAEE. Only 7 compounds —5 bases (adenine, guanine, hypoxanthine, xanthine and uracil), 1 sugar (mannitol), and 1 cinnamic acid derivative (*p*-coumaric acid)— were tentatively identified in both samples. Therefore, 17 compounds —*viz.,* 7 fatty acids and derivatives, including palmitic and stearic acid, 2 fatty amides (oleamide and 13-docosenamide), 3 indoles, 1 quinoline derivative, 2 amino acids, 1 phenol derivative and 1 benzoic acid derivative— were exclusively identified in the ethyl acetate extract and 83 in AbAEE. Fig. 5C shows the Venn diagram comparing the detection coverage in AbAEE analysis *versus* that of the ethyl acetate extract.

Only 3 out of the 24 compounds identified in the ethyl acetate extract were also identified in the hexane and dichloromethane extracts. Stearic acid and 2 fatty amides (oleamide and 13-docosenamide) were the compounds common to the three extracts. Palmitic acid was also identified in hexane, while 8 out of the 24 compounds identified in

the ethyl acetate extract were also identified in the dichloromethane extract, but no in the hexane extract. Four fatty acids derivatives (3-hydroxycapric acid, 5,8-DiHODE, 9,10-dihydroxystearic acid and 9,10-epoxy-18-hydroxystereate), formyl indole, 2,5-dihydrophenylalanine, adenine, and *p*-aminophenol were the compounds common to dichloromethane and ethyl acetate extracts. Among the 12 compounds tentatively identified in ethyl acetate but no in hexane or dichloromethane were 3 purines, 2 indoles, 1 pyrimidine derivative, 1 fatty acid derivative, 1 amino acid, 1 quinoline derivative, 1 monosaccharide, 1 benzoic acid derivative, and 1 cinnamic acid derivative. Purine bases (hypoxanthine, xanthine and guanine), and a pyrimidine derivative (uracil) were found in the ethyl acetate extract, in agreement with the literature (Kaneko, Aoyagi, Fukuuchi, Inazawa, & Yamaoka, 2014; Papaspyridi *et al.*, 2012).

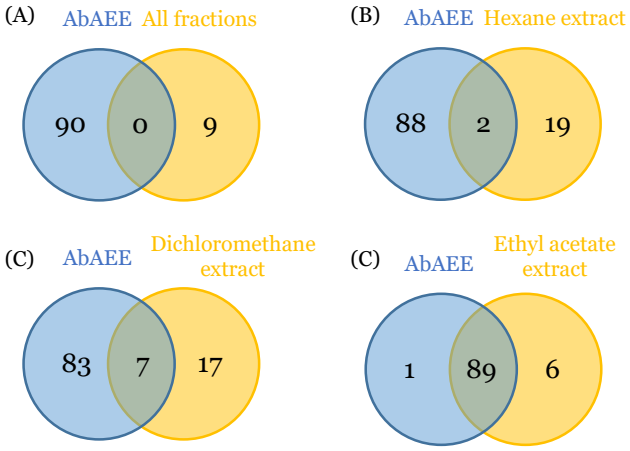


Fig. 5. Venn diagrams comparing the compounds identified in *A. bisporus* aqueous enzymatic extract (AbAEE) with those in the **(A)** hexane extract, **(B)** dichloromethane extract, **(C)** ethyl acetate extract and **(D)** remaining aqueous phase with those in AbAEE.

Other compounds identified in ethyl acetate extracts were indoles (indole-3-carboxylic acid (Qian *et al.*, 2014), and 5-hydroxyindolepyruvate). While indole-3-carboxylic acid is an aromatic compound produced by fungi (Qian *et al.*, 2014) valuable by pharmaceutical and food industries, 5-hydroxyindolepyruvate had not previously been identified in mushroom. Phenolic compounds such as benzoic acid (salicylic) and cinnamic acid derivatives (*p*-coumaric), which focus much attention due to their antioxidant activity,

also were tentatively identified in ethyl acetate extracts. These compounds had previously been identified by both LC–MS/MS and spectrophotometric tests in wild growing edible mushrooms (Nowacka *et al.*, 2014). D-Mannitol, found in ethyl acetate extracts, is considered as a taste-active component in mushroom sugars and polyols (Pei *et al.*, 2014). 9,10,13-TriHOME is a metabolite of linoleic acid (an essential fatty acid) that had previously been identified in plants as rice (Chang *et al.*, 2012), but no in mushrooms. Other compounds no previously identified in mushroom were anthranilic acid and 5,6-dihydroxy-3-methyl-2-oxo-1,2,5,6-tetrahydroquinoline.

3.6. Comparison of the metabolites tentatively identified in the high polar fraction (remaining aqueous phase) with AbAEE and the other extracts

The analysis of the aqueous phase remaining after the extraction sequence allowed identification of 95 compounds *versus* 90 in AbAEE. Among them, 89 were common to both samples because of their aqueous nature. The fact that even after the extraction sequence more metabolites were identified in the remaining aqueous phase than in AbAEE can be explained by the higher dilution of the latter (5 times more diluted). Comparison between these two samples showed that 6 compounds —three amino acids (ornithine, lysine and arginine), 1 dicarboxylic acid (succinic acid), 1 monosaccharide (D-glucosamine 6-phosphate) and 1 phenol (*p*-aminophenol)— were exclusively identified in the remaining aqueous phase and 1 compound (uracil) was identified in AbAEE, but no in the remaining aqueous phase (this compound was also identified in the ethyl acetate extract). Fig. 5D shows the Venn diagram comparing the metabolite coverage in AbAEE and in the aqueous solution after the extraction sequence. As can be seen, fractionation favors coverage as a less diluted sample allows identification of some low concentrated compounds. The removal of no polar and medium polar compounds during fractionation avoids potential ionization suppression in the remained aqueous phase caused by these compounds. Only 2 (adenine and *p*-aminophenol) out of the 95 compounds identified in the remaining aqueous phase were also identified in ethyl acetate and dichloromethane extracts. 4-Hydroxybenzaldehyde was also identified in dichloromethane extract, while 5 out of the 95 compounds identified in the remaining aqueous phase were also identified in the ethyl acetate extract but no in the dichloromethane extract. Three purines (guanine, xanthine, and hypoxanthine), mannitol and *p*-coumaric acid were the compounds common to the ethyl acetate extract and the remaining aqueous phase.

Among the 87 compounds identified in the remaining aqueous phase, but no in dichloromethane and ethyl acetate extract were 1 alkaloid derivative, 2 alkylamines, 1 pyrroline, 37 amino acids and derivatives, 2 peptides, 6 carboxylic acids and derivatives, 6 monosaccharides, 2 disaccharides, 6 sugar acids and derivatives, 1 purine derivative, 1 purine nucleoside derivative, 8 purine nucleotides and derivatives, 6 pyrimidine nucleotides and derivatives, 1 pyridine derivative, 2 fatty acids and conjugates, 2 phospholipids and derivatives, 1 furan and 2 no metal oxoanionic compounds.

Amino acids constitute a family of compounds identified by combining positive and negative ionization modes in the remaining aqueous phase. Two classes of amino acids were detected in this study: proteinogenic and no proteinogenic amino acids. Among the former are essential and no essential amino acids. The identified essential amino acids include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, which had previously been identified in mushroom using different protocols (O'Gorman, Barry-Ryan, & Frias, 2012). No essential amino acids such as tyrosine, aspartic acid, arginine, glutamate, glutamine, proline, serine, and asparagine had also been previously identified in mushroom (Mdachi, Nkunya, Nyigo, & Urasa, 2004), as did no proteinogenic amino acids such as ornithine (Valentaõ *et al.*, 2005), citrulline, cystathionine (Ming, Li, Huo, Wei, & Chen, 2014), pipecolic acid (Kiyoto *et al.*, 2008), and 2-aminobutyric acid (Dembitsky, Terent'ev, & Levitsky, 2010). Interesting amino acids such as ergothioneine and carnitine had also been previously found in mushroom (Kim *et al.*, 2009; Weigand-Heller, Kris-Etherton, & Beelman, 2012). Ergothioneine (an amino acid characteristic of fungi that cannot be produced by human cells) is a natural antioxidant with potential applications as a food additive (Cremades *et al.*, 2015; Weigand-Heller *et al.*, 2012;).

Regarding carnitine, about 80% of this compound is delivered by food. Intake of products rich in L-carnitine is of particular interest for people who are on a slimming diet as this compound may reduce the body weight. Agaritine, saccharopine and pyroglutamic acid are other amino acids found in the remaining aqueous phase, in agreement with previous studies (Cho, Jang, Park, & Park, 2008; Lu, Zeng-Tao, Yi, Xiao-Yan, & Yin-Bing, 2012; Tomoko, Hiroko, Yasuo, & Tatsuyuki, 1999); while other amino acids and derivatives such as 3-amino-2-naphthoic acid, 3-amino-3-(4-hydroxyphenyl)propanoate, 5-aminopentanoate, acetylcarnitine, cycloleucine, 2-aminoadipic acid, LL-2,6-diaminoheptanedioate, N2-acetyl-L-ornithine, N6-acetyl-L-2,6-diaminoheptanedioate, N-

carbamylglutamate and Nε-acetyl-L-lysine had not been previously identified in mushroom. Dipeptides such as γ-D-glutamylglycine and oxidized glutathione had been previously identified in mushroom (Maity *et al.*, 2014; Oka, Tsuji, Ogawa, & Sasaoka, 1981).

Other family detected in the remaining aqueous phase was sugars, among which stand essential monosaccharides such as α-D-glucose and no essential such as D-glucose 6-phosphate, D-glucosamine 6-phosphate, D-mannitol 1-phosphate, sedoheptulose-7-phosphate or sedoheptulose; disaccharides such as cellobionic acid or α-1,5-L-arabinobiose; and sugar acids and derivatives such as galactonic acid, glucuronic acid, glyceric acid, xylonate, glycerol-2-phosphate or 2-dehydro-3-deoxy-L-arabinonate. Among these compounds only two (glucose and sedoheptulose) had previously been described in mushroom (Muszyńska *et al.*, 2013b). Other sugars such as glucuronic acid had not been previously found in free form, but mushroom polysaccharides are formed from these sugars (Wasser, 2002). Many of the sugars identified in the remaining aqueous phase had not previously found in mushroom because most authors only had measured the total amount of sugars (Barros *et al.*, 2008).

Lipids, which can be metabolized as a primary energy source, constitute other super class of compounds identified in the remaining aqueous phase. Two classes of lipids were detected in this study: fatty acids and conjugates, and phospholipids and derivatives. Among the identified fatty acids and conjugates are 2-hydroxyisocaproic acid and ε-capramine, which had not previously been identified in mushroom. Phosphocholine and glycerophosphocholine were the only phospholipids detected in the remaining aqueous phase, but there are in the literature no reports about the presence of these compounds in mushroom.

Carboxylic acids were identified in AbAEE by the negative ionization mode. A monocarboxylic acid (lactic acid) was identified in mushroom, in agreement with the literature that detected this compound by ^{1}H nuclear magnetic resonance spectroscopy (Park *et al.*, 2013). Dicarboxylic acids such as malic, succinic and 2,2-dymethyl succinic were also confirmed in this analytical sample (Barros, Pereira, & Ferreira, 2013). Among them, the last was the only compound that had not previously been found in mushroom. Citric acid was the only tricarboxylic acid detected in the remaining aqueous phase, in agreement with previous studies (Barros *et al.*, 2013). A carboxylic acid derivative such as 3-hydroxy-3-methyl-glutaric acid was also identified in the remaining aqueous phase, but this compound had not previously been found in mushrooms.

Metabolites involved in the degradation of purine nucleosides such as uric acid were also detected in AbAEE, but uric acid had not previously been identified in mushrooms.

Nucleotides, widely used in all types of cells for energy transfer in metabolic processes, constitute other family of compounds identified in the remaining aqueous phase. Two classes of nucleotides were detected in this study: purines and pyrimidines. Among the identified purine nucleotides —monophosphatenucleotides (AMP) (Ranogajec, Beluhan, & Šmit, 2010), 3´-AMP, N6-(1,2-dicarboxyethyl)-AMP, GMP (Mizuno, 1995), cGMP and diphosphatenucleotides (ADP) and GDP (Cheng *et al.*, 2012), GDP-mannose)—, those no previously identified in mushroom are N6-(1,2-dicarboxyethyl)-AMP, cGMP and GDP-mannose. Six pyrimidine nucleotides and derivatives with uracil (UMP, 2,3-cUMP, UDP-D-xylose, UDP-glucose, UDP-glucuronic acid and UDP-N-acetylglucosamine) were also identified in the remaining aqueous phase, but only UMP had previously been identified in mushroom (Ranogajec *et al.*, 2010). Xanthosine was the only purine nucleoside derivative found, and it had previously been identified in mushroom (Ranogajec *et al.*, 2010).

Alkylamines such as spermidine and pantothenic acid had also been previously identified in mushroom (Mukhopadhyay & Guha, 2015) —the latter is a water-soluble vitamin essential for humans (Zempleni, Suttie, Gregory III, & Stover, 2013). Other water-soluble vitamin found in the remaining aqueous phase was nicotinic acid (Papaspyridi *et al.*, 2012).

The rest of the compounds detected in the remaining aqueous phase (2-methylpyrrolidine, 1-pyrroline, 2-furoic acid, phosphate and phosphite) have been identified in mushroom for the first time.

4. Conclusions

The *in vitro* inhibitory activity of AgAEE against the HCV protease NS3 makes the former a potential nutraceutical suitable for use as it is or as component of new functional foods, that could be used for prevention of HCV infection.

Fractionation based on LLE with different extractants has been used for drastic improvement of the tentative identification coverage of metabolites in AbAEE. The possibility of either dilution or preconcentration of the fractions decisively contributed to

improve coverage. Table 1 shows all the tentatively identified metabolites, where those identified for the first time are highlighted in bold. In short, 55 out of the 123 tentatively identified metabolites have been identified in AbAEE and extensively in the edible mushroom *A. bisporous* (WBM) for the first time. The results confirm that fractionation improves the characterization of the WBM (*A. bisporus*) metabolome as some of the metabolites identified for the first time have been found in organic fractions —which can be attributed to the preconcentration step. Nevertheless, most of them have been found in both the AbAEE and remaining aqueous fraction, which strongly support that LC–QTOF MS/MS is a good strategy to analyze this fungus extracts.

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Supplementary material

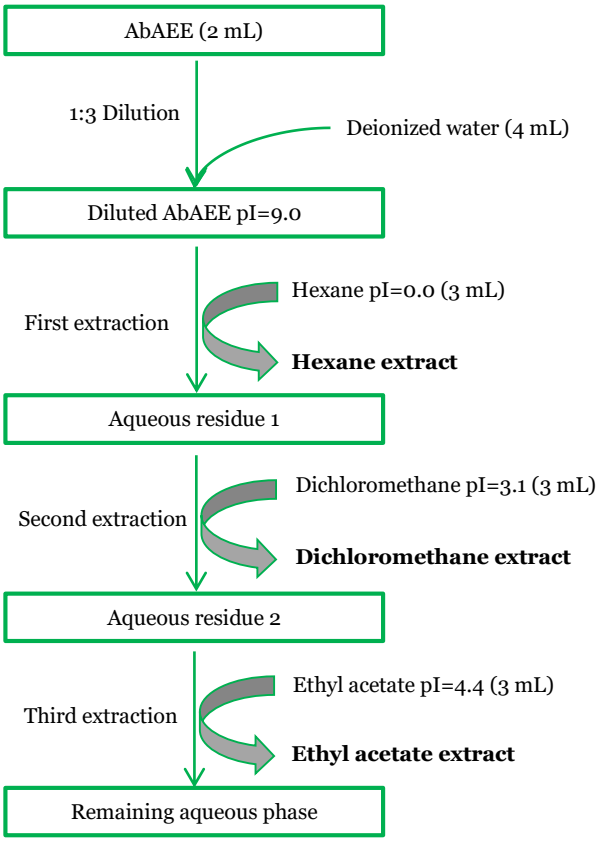

Fig. S1. Fractionation of *A. bisporus* aqueous enzymatic extract (AbAEE) by sequential liquid–liquid extraction with three extractants.

Chapter VI

Untargeted characterization of extracts from *Cannabis sativa* L. cultivars by gas and liquid chromatograpy coupled to mass spectrometry in high resolution mode

Untargeted characterization of extracts from *Cannabis sativa* L. cultivars by gas and liquid chromatography coupled to mass spectrometry in high resolution mode

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Abstract

Elucidation of *Cannabis* composition is required to evaluate the potential of this plant for pharmacological uses, but also for implementation in breeding programs with agronomical purposes. The aim of the present study was to develop a method for untargeted analysis of polar and no polar *Cannabis* extracts. For this purpose, extracts from 17 cultivars of *Cannabis sativa* L. were analyzed by gas chromatography–time-of-flight/mass spectrometry (GC–TOF/MS) and liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC–QTOF MS/MS) in high resolution mode. One hundred sixty-nine compounds were identified in the extracts by searching MS and MS/MS information. Among identified families, there were mainly cannabinoids, terpenoids, lipids and flavonoids, but also some interesting compounds such as amino and organic acids, among others. Relative contents of terpenoids and cannabinoids in the same cultivars grown in greenhouse and field were compared. Compositional differences in the profile of terpenoids and cannabinoids between both types of grown conditions were found.

Keywords: *Cannabis sativa* L. metabolome, gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, cannabinoids, terpenoids, *Cannabis sativa* L. varieties.

1. Introduction

The *Cannabaceae* is a family of flowering plants that includes 10 genera and 117 accepted species [1]. Among them, *Cannabis* has been the most widely studied because of its pharmacological and psychoactive properties. *Cannabis* is used in the treatment of disorders such as migraine, spastic and pain disorders, and glaucoma as ocular hypotensive. Countries such as Canada, Germany, Israel, the Netherlands and more than 50% of the United States have fully authorized the medical application of herbal *Cannabis* [2]. The pharmacological properties of *Cannabis* have been traditionally ascribed to the presence of cannabinoids [3–5]. However, there are some evidences regarding the differential effects of *Cannabis* preparations as compared to only cannabinoids [6].

Most analytical studies on characterization of *Cannabis* plants were targeted at the detection of two chemical families: cannabinoids and terpenoids. Cannabinoids are a family of compounds produced from fatty acids and isoprenoids precursors that are specific of *Cannabis*. They are primarily found in the resin produced in the glandular trichomes of pistillate (female) inflorescences [7]. On the other hand, terpenoids are other family of isoprenoids compounds found in plants, which are responsible for protecting them from predators and attract pollinating insects for propagation. The variety of monoterpenes and sesquiterpenes contained in *Cannabis* resin contribute to the scent of flowers and to the unique flavor qualities of *Cannabis* products. [7]. Pharmacologically, they have been implicated in influencing the properties of the cannabinoids, possibly by an entourage effect [8].

The analysis of both chemical families has been addressed with different analytical platforms [9], mainly by a combination of gas chromatography and liquid chromatography coupled to mass spectrometry (GC–MS, LC–MS) [10–13]. Concerning cannabinoids, a recent method based on GC–tandem mass spectrometry (MS/MS) allowed detecting seven trialkylsilylated cannabinoids in *Cannabis*-type ruderalis plant tissues [14]. Derivatization is a frequent step prior to GC separation of cannabinoids. Thus, different derivatization agents have been tested for the fast determination of 9 cannabinoids by GC–MS [12].

LC–MS/MS has also provided optimum response in cannabinoids analysis. A recent research allowed detecting 94 phytocannabinoids in 10 species by LC–MS/MS with electrospray ionization [15]. This method was used to profile the most commonly used *Cannabis* plants prescribed to patients in Israel. In other study, 6 cannabinoids

[cannabidiol (CBD), δ9-tetrahydrocannabivarin (Δ9-THCV), cannabigerol (CBG), cannabinol (CBN), δ9-tetrahydrocannabinol (Δ9-THC) and δ9-tetrahydrocannabinolic acid (Δ9-THCA)] from 30 strains of *Cannabis sativa* were determined by LC–MS [16].

Terpenoids are mainly analyzed by GC–flame ionization detection (GC–FID) or GC–MS based on their high volatility [17,18]. In a recent study, 16 terpenoids were identified by GC–MS and quantified by GC–FID [19]. Additionally, some studies have been focused on the combined analysis of terpenoids and cannabinoids. In one of them, 23 terpenoids (both monoterpenoids and sesquiterpenoids), and 9 cannabinoids were quantitatively determined in 11 *Cannabis* varieties by GC–FID [20]. The evolution of 8 major cannabinoids and 28 terpenes during the growth of *Cannabis sativa* plants for 22 weeks was studied in another research by LC–diode array detection (HPLC–DAD) and GC–FID, respectively [21].

In addition to cannabinoids and terpenoids, there are scant studies dealing with other compounds in *Cannabis*. Five phenolic compounds (quercetin, gallic acid, *p*-coumaric acid, *m*-coumaric acid and cinnamic acid) were identified in *Cannabis* plants by LC–UV spectrophotometry [22].

The development of untargeted strategies for characterization of *Cannabis* plant material is so far limited. In a recent study, 9 amino acids, 6 sugars, 4 cannabinoids, 4 carboxylic acids and choline were identified in chloroform and aqueous extracts of *Cannabis sativa* trichomes by nuclear magnetic resonance (NMR) spectroscopy [23]. Also, 18 compounds such as pamine and tyramine derivatives, among others, were identified in hemp (*Cannabis sativa* L.) seeds by NMR [24]. LC–MS was the selected platform for identification of 8 compounds in *Cannabis sativa* L. leaves to evaluate the effect of UV-C radiation on their metabolites [25].

Clearly, more research on *Cannabis* composition is demanded to assess its potential for pharmacological use, but also to implement breeding programs with agronomical purposes. With these premises, the aim of the present study was to develop a method for untargeted metabolomics analysis of polar and no polar *Cannabis* extracts. For this purpose, two analytical platforms —gas chromatography–time-of-flight/mass spectrometry (GC–TOF/MS) and liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC–QTOF MS/MS)— were used to maximize the detection coverage.

2. Experimental

2.1. Reagents and chemicals

LC–MS grade methanol and *n*-hexane from Sigma–Aldrich (St. Louis, MO, USA) were used for standards preparation and sample treatment. LC–MS grade acetonitrile (ACN) from Thermo Fisher Scientific (Pittsburgh, PA, USA) and MS grade formic acid (FA) from Scharlab (Barcelona, Spain) were used to prepare the chromatographic mobile phases for LC–MS/MS analysis. Deionized water (18 mΩ · cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the aqueous solutions.

Pyridine, bis-(trimethylsilyl) fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were the solvent and silylation agents in the derivatization step. MS-grade perfluorotri-*n*-butylamine (PFTBA) from Agilent Technologies (Santa Clara, CA, USA) was used for daily mass calibration. A standard mixture containing 10 linear alkanes from C10 to C40 designed for performance tests in GC from Sigma–Aldrich was used to establish the retention index (RI) calibration model. Standards of kaempferol, kaempferol-7-glucoside, vitexin, vitexin-2-rhamnoside, quercetin, quercetin-3-galactoside, luteolin-6-glucoside (homoorientin), diosmetin, apigenin, rutin, naringenin, luteolin, naringin, procyanidins A2, B1 and B2, maltose, sucrose, lactose, glucose, stearic acid and pyroglutamic acid from Sigma–Aldrich and Extrasynthese (Lyon, France) were used to confirm the identification of flavonoids, sugars and other compounds in the extracts.

Standards of cannabidivarin (CBDV), Δ9-THCV, CBD, cannabicyclol (CBL), cannabidivarinic acid (CBDVA), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabichromene (CBC), δ8-tetrahydrocannabinol (Δ8-THC), Δ9-THC, CBG, CBN and THCA from Cerilliant (Darmstadt, Germany) were used to confirm the identification of cannabinoids. Finally, standards of α-pinene, (-)-β-pinene, (-)-camphene, sabinene, β-myrcene, α–phellandrene, 3-carene, p-cymene, limonene, eucalyptol, *trans*-β-ocimene, β-ocimene, γ-terpinene, linalool, (+)-fenchol, (+)-camphor, isopulegol, (-)-borneol, menthol, α-terpineol, dihydrocarveol, nerol, citronellol, R-(+)-pulegone, *cis*-citral, geraniol, *trans*-citral, longipinene, β-elemene, cedrene, *trans*-(-)-caryophyllene, aromadendrene, valencene, *cis*-nerolidol, farnesene, caryophyllene oxide, guaiol, β-eudesmol, α-bisabolol,

trans-trans-farnesol, *cis*-phytol and *trans*-phytol from Sigma–Aldrich were used to confirm the identification of terpenoids.

2.2. Instruments and apparatus

An Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer was used for LC–MS/MS analysis. This approach was equipped with a dual electrospray ionization (ESI) source (Santa Clara, CA, USA) for simultaneous spraying of a mass reference solution to calibrate continuously detected *m/z* ratios.

Complementarily, an Agilent 7890A Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with electron impact (EI) source was also used for analysis.

Both instrumental configurations were controlled through MassHunter GC–QTOF and LC–QTOF acquisition softwares (version B.06 and B.07, respectively, from Agilent Technologies).

2.3. Samples and preparation of extracts

Samples formed by leaves and inflorescences collected from 17 cultivars of *Cannabis* plants (3 biological replicates) were supplied by Phytoplant Research S.L., which is authorized by the Spanish Agency of Medicines and Medical Devices (AEMPS). Some of these plants are asexually propagated medicinal varieties registered by the company in the Community Plant Variety Office (CPVO) (http://cpvo.europa.eu/en) and identified with denomination proposals and application numbers: 'Aida', 50012; 'Beatriz', 50014; 'Divina', 50045; 'Juani', 50011; 'Magda', 50011; 'Mati', 50015; 'Moniek', 50008; 'Octavia', 50016; 'Pilar', 50009; 'Sara', 50007 and 'Theresa', 50009; while other genotypes and hybrids, obtained as a result of an internal breeding program, and modern industrial hemp varieties were also analyzed. Table 1 lists the cultivars selected for this study, the growing conditions and locations. Five cultivars were only grown indoor with photosynthetic active radiation (PAR) light exposition according to the legislation due to their high THC concentration. Other cultivars were grown in high tunnels in field or in high technology greenhouses. Irrigation doses, plant densities and sowing times were applied to each cultivar to maximize

the agronomic performance. Four cultivars (namely, 'Aida', 'Juani', 'Pilar' and 'Theresa') were grown in field and in greenhouse. They were used to evaluate the influence of the growing conditions on the composition of extracts.

Table 1. List of the cultivars and growing conditions.

Code	Cultivar	Growing conditions
Theresa SCAI-6	Theresa	Greenhouse
Theresa IFAPA 2016	Theresa	Field
Pilar flores SCAI-6	Pilar	Greenhouse
Pilar IFAPA 2016	Pilar	Field
Aida SCAI-5	Aida	Greenhouse
Aida IFAPA 2016	Aida	Field
Sara IFAPA 2016	Sara	Field
Juani SCAI-5	Juani	Greenhouse
Juani IFAPA 2016	Juani	Field
Octavia IFAPA 2016	Octavia	Field
Mati (flor + hoja)	Mati	Indoor
Beatriz (flor + hoja)	Beatriz	Indoor
Magda 34/2 flor estándar	Magda	Indoor
Moniek flor standariz	Moniek	Indoor
Divina	Divina	Indoor
H6 IFAPA 2016	H6	Field
H7 IFAPA 2016	H7	Field
Ermes (Appendino) 2017	Ermes	Field
Carma (Appendino) 2017	Carma	Field
Carmagnola (Appendino) 2017	Carmagnola	Field
Futura (Appendino) 2017	Futura	Field

Samples of the selected plant material were processed using the same protocol. A 30 cm part of each plant containing leaves and at least one female inflorescence was collected to get around 30 g of fresh vegetal material. Plant samples were oven dried at 40

ºC (J. P. Selecta model Conterm 2000210, Barcelona, Spain) for 72 h, and the stems and seeds of more than 2 mm in size were removed.

Powdered plant materials (100 mg) were extracted with 5 mL of *n*-hexane or methanol for GC or LC analysis, respectively, placed in an ultrasound bath at 37 kHz and 800 W, for 20 min, and centrifuged for 5 min at 2121×*g*. Then, the supernatants were collected and stored at –80 ºC until analysis.

2.4. Sample preparation

Different dilutions of the raw methanol and hexane extracts were tested prior to analysis. For derivatization, 300 µL of each 1:1000 diluted hexane extract was evaporated to dryness, reconstituted by 270 µL of BSTFA + TMCS (98:2 v/v) + 30 µL of pyridine, and maintained at 37 ºC for 1 h. Finally, the derivatized mixture was located in a new glass insert for analysis.

2.5. LC–QTOF MS/MS analysis

LC separation and QTOF detection were used for analysis of the methanol extracts. Chromatographic separation was performed using a C18 reverse-phase analytical column (Mediterranean, 25 cm×0.46 cm i.d., 3 µm particle size) from Teknokroma (Barcelona, Spain), which was thermostated at 34 ºC. The mobile phases were water with 5% ACN (phase A) and ACN with 5% water (phase B) both with 0.1% FA as ionization agent. The LC pump was programmed at a flow rate of 0.7 mL/min with the following elution gradient: 4% phase B as initial mobile phase was kept constant since min 0 to 1; then, the percentage of phase B was increased from 4 to 20% since min 1 to 5, from 20 to 70% of phase B since min 5 to 10, from 70 to 90% of phase B since min 10 to 20, from 90 to 100% of phase B since min 20 to 32 and finally 100% phase B was kept constant since min 32 to 42. A post-time of 10 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 5 µL and the injector needle was washed between injections with 80% methanol for 10 s. The parameters of the electrospray ionization source, operating in negative and positive ionization modes, were as follows: the capillary and fragmentor voltage were set at ±3.5 kV and 175 V, respectively; N_2 in the nebulizer flowed at 40 psi; the flow rate and temperature of the N_2 as drying gas were 10 L/min and 325 ºC, respectively.

The instrument was calibrated and tuned according to procedures recommended by the manufacturer. MS and MS/MS data were collected in both polarities using the centroid mode at a rate of 2.4 spectra/s. Accurate mass spectra in MS scan and MS/MS mode were acquired in the *m/z* range 60–1100. The instrument gave typical resolution 15000 FWHM (full width at half maximum) at *m/z* 118.0862 and 30000 FWHM at *m/z* 922.0098. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by using the signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H,1H,3Htetrafluoropropoxy) phosphazine or HP-921] in the positive ionization mode, while in negative ionization mode ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.0007 (formate adduct of HP-921) were used. The samples were injected in auto MS/MS acquisition mode to obtain information on the product ion of the most relevant compounds. The number of precursors selected per cycle was set at 2, with an exclusion window of 0.1 min after two consecutive selections of the same precursor. Two collision energies (20 and 40 eV) were used to obtain the fragmentation information for each entity. Three replicates of each analytical sample were analyzed to improve the quality of the results.

2.6. GC–TOF/MS analysis

The hexane extracts were analyzed by GC–TOF/MS by setting the ionization voltage at 70 eV. Chromatographic separation was carried out with a fused silica DB-5MS-UI 30 m×0.25 mm i.d.×0.25 μm film thickness capillary column. The GC oven temperature program started at 50 °C with several consecutive temperature ramps: from 2 °C/min to 104 °C (27 min held), from 20 °C/min to 120 °C (0.8 min held), from 4 °C/min to 160 °C (10 min held), from 25 °C/min to 232 °C (2.9 min held), from 1.5 °C/min to 242 °C (6.7 min held), from 2 °C/min to 250 °C (4 min held), from 25 °C/min to 300 °C (2 min held), temperature that was kept constant for 10 min. A post-run time was programmed for 4 min up to 310 °C to assure complete elution of the injected sample. One μL of sample was injected by splitless injection mode at 250 °C using ultrapure grade helium as carrier gas at 1 mL/min. The transfer line, ion source and quadrupole temperatures were set at 305, 305 and 200 °C, respectively. A solvent delay of 3 min was used to prevent damage of the ion source filament. The TOF detector was operated at 7 spectra/s in the mass range *m/z* 50–750 and the resolution was 7500 (full width half maximum, FWHM) at *m/z* 501.9706. Daily mass calibration was performed with PFTBA. All samples were injected in triplicate.

In addition, the described method was adapted for the analysis of the derivatized solutions using pulse divided injection (1:10 split ratio) of one μL of the given solution at 250 °C, and changing the solvent delay of the original method to 5.5 min to avoid filament damage.

2.7. LC–QTOF MS/MS data processing and statistical analysis

MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to process the data obtained by LC–QTOF in auto MS/MS mode. Treatment of raw data files started by extraction of potential molecular features (MFs) with the suited algorithm included in the software. For this purpose, the extraction algorithm considered all ions with maximum charge state of 2 exceeding 400 and 500 counts for negative and positive ionization modes, respectively. These cut-off values were established taking into account the chromatographic background noise. Additionally, the isotopic distribution to consider MFs as valid should be defined by two or more ions (with a peak spacing tolerance of *m/z* 0.0025, plus 7.0 ppm in mass accuracy). In the search criteria for MFs, ions and adducts formation in the positive (+ H, + Na) and negative (– H, + HCOO, + Cl) ionization modes, as well as neutral loss by dehydration were included to identify features corresponding to the same potential metabolite. Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities and other contaminants after analysis of a blank of pure methanol.

Identification of the compounds was supported on MS and MS/MS information and search in the METLIN MS and MS/MS databases (http://metlin.scripps.edu), the Human Metabolome Database (HMDB, 4.0 version), the Metfrag database (https://msbi.ipb-halle.de/MetFrag/) based on mass spectra of metabolites generated by *in silico* fragmentation, and on a metabolites library generated in previous research [26] using in all cases the MFs obtained in the present research. In addition, standard solutions were used to confirm the presence of identified compounds.

In a next step, the "Batch Targeted Feature Extraction" algorithm from the MassHunter Workstation software (version B8.00 Profinder, Agilent Technologies, Santa Clara, CA, USA) was used to extract and align peaks, from all chromatograms, that matched a database of the identified metabolites. The isotopic distribution for a valid feature had to

be defined by two or more ions, with a peak spacing tolerance of 0.0025 *m/z*, plus 7.0 ppm. The features were aligned by using a tolerance window of 0.30 min and a mass accuracy of 10 ppm for retention times (RTs) and *m/z* values, respectively, across all data files. Each extracted ion chromatogram was individually examined, and potentially incorrect assignments and integrations were manually verified. A table with the peak area of all identified compounds in the different samples was thus obtained.

2.8. GC–TOF/MS data processing and statistical analysis

Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process the data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to treat all data files. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of MFs considered as potential compounds defined by the *m/z* value of one representative ion for each chromatographic peak and its RT. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions, the accuracy error at 50 ppm and the window size factor at 200 units. The background spectrum was subtracted to the spectrum obtained from each sample before its deconvolution.

Tentative identification of compounds was firstly performed by searching MS spectra in the NIST11 database. Only identifications with a match factor and a reverse match factor higher than 700 were considered. The RI values included in the National Institute of Standards and Technology (NIST) database also supported identifications. For this purpose, an RI calibration model for each method was built by plotting the RT obtained by analysis of the alkane standard mixture (C10 to C40 with an even number of carbons) with the chromatographic method used in this research and the RI values provided for each alkane by the NIST database. The equations thus obtained were used to experimentally estimate the RI value for each identified compound, which must be less than 100 units different from the theoretical value provided by the NIST. This database does not contain high resolution MS information as provided by the TOF detector; therefore, a third step was included to validate identification of each compound by high resolution MS. Thus, the molecular formula for the tentative ion $[M]^+$ and the most intense fragments for each MF should fit the NIST identification by setting a cut-off value in mass accuracy of 10 ppm.

Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities and other contaminants after analysis of a blank of pure hexane.

In addition, the multistandard solutions of terpenoids and cannabinoids were injected to confirm their identification in the extracts.

Finally, the MassHunter Workstation software (version 7.0, Quantitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to reintegrate all metabolites found in all analyzed samples, using the corresponding characteristic quantifier and qualifiers. A table with the peak area of all identified compounds in the different samples was obtained and combined with those from LC–MS/MS data.

PASW Statistics 18 (version 18.0.0) was used to statistically analyze the differences in the content of cannabinoids and terpenoids among different varieties of cultivars and cultivation.

3. Results and discussion

3.1. Identification of compounds in Cannabis

Polar extracts of *Cannabis* were analyzed by LC–QTOF MS/MS, while no polar extracts were characterized by GC–TOF/MS. 1:40 (v/v) dilution factor was the most suitable for analysis of polar extracts in positive ionization mode, while 1:100 (v/v) was selected for negative polarity. In the case of no polar extracts, 1:50 (v/v) dilution factor was the most suitable for direct analysis by GC–TOF/MS, while 1:1000 (v/v) was adopted for analysis after derivatization. Supplementary Figs. 1 and 2 show the total ion chromatograms (TICs) provided by LC–MS/MS analysis in the two ionization modes and GC–MS, for the derivatized and underivatized extracts.

Tentative identification of MFs using MS and MS/MS data in high resolution mode led to a total number of 169 compounds with an accuracy error below 8.4 ppm. The identified compounds and the main parameters that support the identification are listed in Supplementary Tables 1-to-3. Most of the compounds were identified in the no polar extract, with special emphasis on neutral cannabinoids, terpenoids and lipids. On the other

hand, most of the compounds detected in the polar extract were cannabinoids and terpenoids, but, in addition, a high number of flavonoids were exclusively identified in the polar extract. Other minor families were also detected in *Cannabis* extracts, hydrocarbons and benzenoids in no polar extracts and carbohydrates in polar extracts.

Compounds reporting the most intense chromatographic peaks both in the polar and no polar extracts of *Cannabis* were mainly cannabinoids. Some terpenoids such as limonene, *trans*-(-)-caryophyllene, caryophyllene, β-myrcene and α-pinene were also detected with high intensity in the no polar extracts, in agreement with a previous study [27]. Additionally, some long-chain fatty acids such as C16:0 and C18:0 also provided high intense peaks in the derivatized extracts.

The chemical distribution of identified compounds was as follows: 22 cannabinoids, 70 terpenoids, 31 lipids, 16 flavonoids, 6 amino acids, 5 organic acids, 4 benzenoids, 4 organic oxygen compounds, 3 hydrocarbons, 3 carbohydrates, 2 organoheterocyclic compounds, 2 organonitrogen compounds and 1 alkaloid. Among them, 134 metabolites were identified in the no polar extracts by GC–TOF/MS, while 46 compounds were identified in the polar extracts by LC–QTOF MS/MS. Eleven of the cannabinoids identified by LC–QTOF MS/MS in the polar extracts where also identified in the no polar extracts by GC–TOF/MS. Supplementary Tables 1 and 2 show the list of the 134 compounds identified in the no polar extracts, which included mainly cannabinoids (20), terpenoids (69) and lipids (27). Most of these compounds (100), especially cannabinoids and terpenoids, were identified in the diluted underivatized extracts; while other 39 compounds (mainly lipids and organic acids) were identified in the derivatized extracts. Five of these 39 compounds were identified in both extracts. The identification of 11 cannabinoids (CBDV, Δ9-THCV, CBD, CBC, Δ8-THC, Δ9-THC, CBG, CBN, CBL, CBDVA and THCA) and 28 terpenes was confirmed with standards.

Complementarily, compounds identified in the polar extracts by LC–QTOF MS/MS included 16 flavonoids, 13 cannabinoids, 6 amino acids, 4 lipids, 2 carbohydrates, 1 alkaloid, 1 indole, 1 organonitrogen compound, 1 terpenoid and 1 urea derivative. Supplementary Table 3 shows the parameters supporting these identifications. Most of them, especially flavonoids and acid cannabinoids, were better identified in negative polarity, while neutral cannabinoids could be easily identified in both polarities. On the other hand, most amino acids and a miscellaneous group of compounds including one glycerophospholipid, one choline derivative, one alkaloid, one indole and one urea

derivative were identified in positive ionization. In this case, the identification of 13 cannabinoids, 9 flavonoids and carbohydrates (CBDVA, CBDV, CBDA, CBG, CBGA, CBD, THCV, CBN, Δ8-THC, Δ9-THC, CBC, CBL, THCA, apigenin, quercetin, quercetin 3-galactoside, kaempferol-7-glucoside, luteolin-6-C-glucoside, diosmetin, vitexin, vitexin-2-rhamnoside, procyanidin B2, maltose and sucrose) was confirmed with standards. The rest of the compounds (22) were tentatively identified by the above-named metabolomics libraries.

3.2. Cannabinoids

Phytocannabinoids are synthesized in *Cannabis* in secretory cells inside glandular trichomes, which are more concentrated in unfertilized female flowers prior to senescence [28,29]. Geranyl pyrophosphate, formed via the deoxyxylulose pathway in *Cannabis* [30], is a precursor of phytocannabinoids (Fig. 1).

The 22 cannabinoids identified in the extracts from *Cannabis*, classified into the main families of phytocannabinoids [31], include: 1) CBG type, 2) CBC type, 3) CBD type, 4) (-)-Δ9-*trans*-THC type, 5) (-)-Δ8-*trans*- THC type, 6) CBL type, 7) cannabielsoin (CBE) type, 8) CBN type, 9) cannabinodiol type, 10) cannabitriol type, and 11) miscellaneous type. Only the last two families did not report any identified compound.

The cannabinoids identified by analysis of no polar extracts were neutral cannabinoids, while two acid cannabinoids were identified after derivatization. Eighteen neutral cannabinoids were identified in the diluted underivatized extracts. The most concentrated neutral cannabinoids CBC, CBD, CBDV, CBG, CBN, Δ8-THC, Δ9-THC and Δ9-THCV were confirmed with standards. Other cannabinoids such as cannabinerol, CBE, CBL, cannabigerovarin (CBGV), isomers of CBC, an isomer of Δ8-THC, an isomer of Δ9-THCV and isomers of Δ9-THC were tentatively identified.

Seven cannabinoids were identified after silylation. Five of them were neutral cannabinoids (CBDV, CBD, CBL, Δ9-THC and CBN), which were also previously identified without derivatization; while THCA and CBDVA were acid cannabinoids additionally identified in no polar extracts. All of them were confirmed with standards.

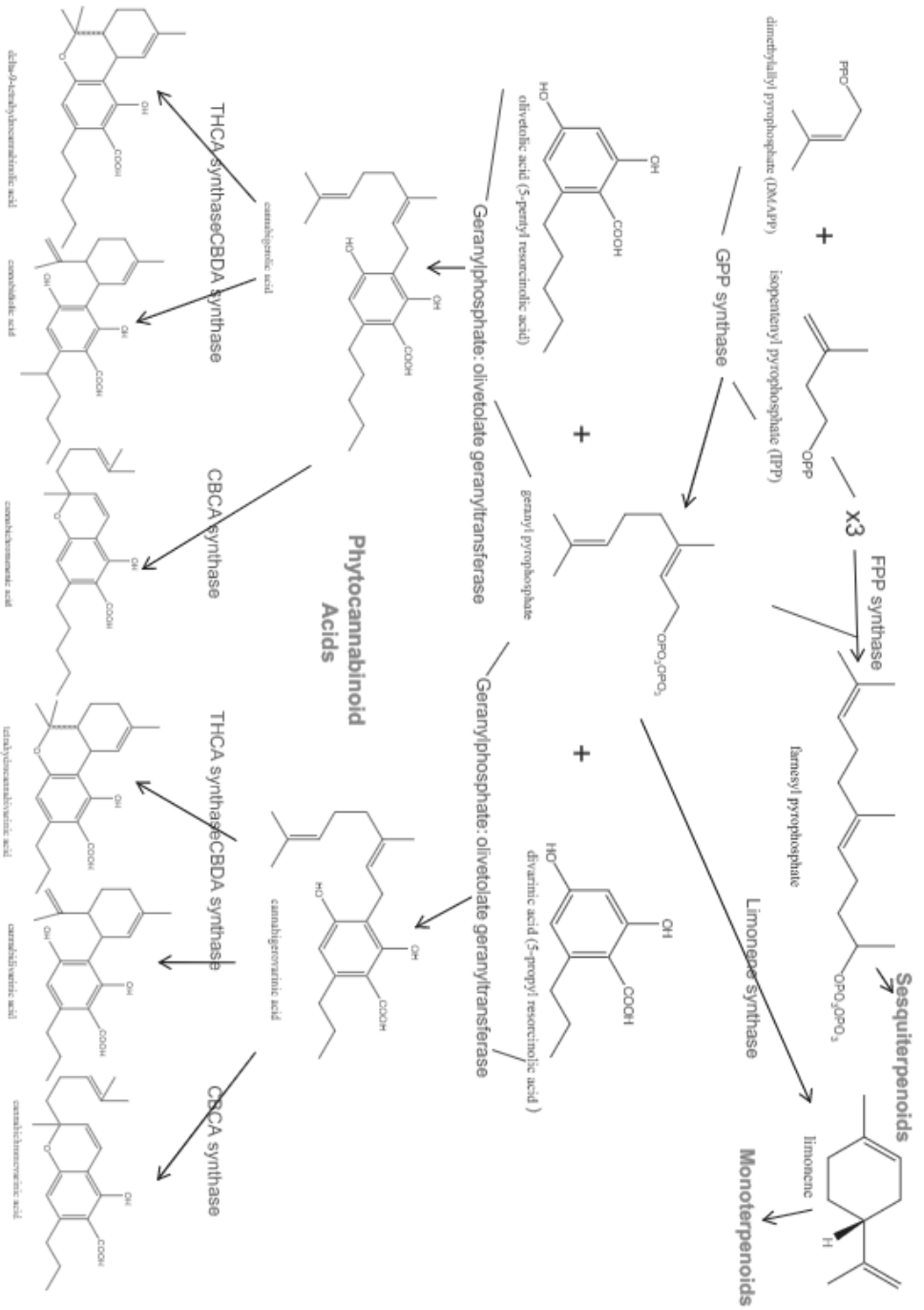

Fig. 1. Phytocannabinoid and *Cannabis* terpenoid biosynthesis [8].

Supplementary Fig. 3 shows the MS spectra obtained after direct analysis of the cannabinoid standards by GC–MS: CBDV (A), Δ9-THCV (B), CBD (C), CBC (D), Δ8-THC (E), Δ9-THC (F), CBG (G) and CBN (H). Among them, only CBDV and Δ9-THCV reported the product ion at *m/z* 203.1079 as representative fragment. Other cannabinoids such as CBD, CBC, Δ8-THC, Δ9-THC gave the product ion at *m/z* 231.1397 as the largest fragment, also present in the MS spectrum of CBG. The fragment at *m/z* 299.2022 was the top product ion in Δ9-THC, while ions at *m/z* 295.1707 and 193.1238 were the representative fragments for CBN and CBG, respectively.

Some minor fragments characteristic for cannabinoids were detected at *m/z* 174.0678, 193.1217, 231.1397 and 271.1692 (Supplementary Fig. 3). In fact, *m/z* 174.0678, 231.1397 and 271.6920 were found in CBDV, Δ9-THCV, CBD, CBC, CBG, Δ8-THC and Δ9-THC. The fragment at *m/z* 193.1238 was found in CBD, Δ8-THC, Δ9-THC, CBG and CBN.

Supplementary Fig. 4 shows the MS spectra of ten tentatively identified cannabinoids [an isomer of Δ9-THCV (cannabinoid 1), an isomer of CBC (cannabinoid 2), CBGV (cannabinoid 3), CBL (cannabinoid 4), an isomer of Δ8-THC (cannabinoid 5), isomers of Δ9-THC (cannabinoids 6 and 9), CBE (cannabinoid 7), isomer of CBC (cannabinoid 8), and cannabinerol (cannabinoid 10)].

The cannabinoids identified in polar extracts by LC–MS/MS were neutral (9) and acid (4) cannabinoids. All of them were confirmed with standards (CBDV, CBG, CBD, THCV, CBN, Δ8-THC, Δ9-THC, CBC, CBL, CBDVA, CBDA, CBGA and THCA). Acid cannabinoids were identified by LC–MS/MS in negative ionization mode as $[M-H]^-$. Also, two neutral cannabinoids, such as CBL and CBN, were better identified by LC–MS/MS in negative ionization mode, while the rest (7) were better identified using positive ionization mode. However, all of them were detected in both polarities, except THCV.

Also, all of them were previously identified by GC–MS, except CBDA and CBGA. CBDA is the precursor of CBD, which gave the most intense peak. It is remarkable that other cannabinoids were also detected by LC–MS/MS in the chromatographic retention interval 19 to 37 min. Cannabinoids fragmentation pattern is quite similar; therefore, identification of cannabinoids based only on MS/MS information is not a reliable process.

3.3. Terpenoids

Terpenoids constituted the second major family of identified compounds in *Cannabis* samples. It is remarkable that volatile terpenoids provide the characteristic aroma to *Cannabis* [8]. Terpenoids are formed also in secretory cells inside glandular trichomes that are much more concentrated in unfertilized female flowers prior to senescence [28,29]. Geranyl pyrophosphate is synthesized in *Cannabis* as a precursor via the deoxyxylulose pathway [30], being the precursor of phytocannabinoids and terpenoids (Fig. 1). Geranyl pyrophosphate may form monoterpenoids such as limonene, among others, in secretory cell plastids, or couple with isopentenyl pyrophosphate in the cytoplasm to form farnesyl pyrophosphate, precursor of sesquiterpenoids. This explains the fact that most identified terpenoids were monoterpenoids and sesquiterpenoids (20 and 65%, respectively). In addition, two diterpenoids and three triterpenoids were tentatively identified.

The identified monoterpenoids included three acyclic monoterpenes (*cis*-β-ocimene, *trans*-β-ocimene and β-myrcene), five monocyclic monoterpenes (*p*-cymene, α-phellandrene, β-terpinene, γ-terpinene and limonene), two monocyclic monoterpene alcohols (menthol and α-terpineol), three bicyclic monoterpenes (α-pinene, β-pinene and camphene), two bicyclic monoterpene alcohols (borneol and fenchol) and one bicyclic monoterpene ether (eucalyptol).

One of the diterpenoids identified, an acyclic diterpene alcohol, was *trans*-phytol, which is present in *Cannabis* extracts as a breakdown product of chlorophyll and tocopherol. It is an aromatic compound used widely in cosmetics as ingredient of many fragrances [32], and studied in medicine due to its antioxidant activity [33]. The other diterpenoid identified was carnosol, a phenolic diterpene, which was the only terpenoid identified by LC–QTOF MS/MS in negative ionization mode due to the presence of hydroxyl groups.

Identified sesquiterpenoids included one acyclic sesquiterpene (farnesene), two acyclic sesquiterpene alcohols (*cis*-nerolidol and *trans-trans*-farnesol), five monocyclic sesquiterpenes (β-elemene, α-curcumene, β-curcumene, β-sesquiphellandrene and α-humulene), one monocyclic sesquiterpene ether (caryophyllene oxide), one monocyclic sesquiterpene alcohol (α-bisabolol), sixteen bicyclic sesquiterpenes (two caryophyllene isomers, *cis*- and *trans*-α-bergamotene, β- and γ-selinene, β- and γ-guaiene, two guaia-

1(10),11-diene isomers, selina-3,7(11)diene, cedrene, valencene and *trans*-caryophyllene, among others), three bicyclic sesquiterpene alcohols (guaiol, β-eudesmol and γ-eudesmol), seven tricyclic sesquiterpenes (two cedrane isomers, di-epi-α-cedrene, a longipinene isomer, (-)-aristolene, aromadendrene and *cis*-thujopsene) and two tricyclic sesquiterpene ethers. Furthermore, one triterpene (squalene) and two triterpene alcohols (α-amyrin and β-amyrin) were identified in the extracts.

Most terpenoids were identified in no polar extracts by GC–TOF/MS, except for carnosol. The identification of terpenoids is a challenging task favored by comparison of their RTs with those provided by standards due to the similarity of MS spectra. Terpenes show representative common fragments due to the structure formed by isoprene units. Some examples of the most characteristic fragments are shown in Supplementary Fig 5. Thus, most terpenes yield fragments at *m/z* values of 51.0235 $[C_4H_3]^+$, 65.0391 $[C_5H_5]^+$, 69.0704 $[C_5H_9]^+$, 77.0391 $[C_6H_5]^+$, 79.0548 $[C_6H_7]^+$, 91.0548 $[C_7H_7]^+$, 93.0704 $[C_7H_9]^+$, 107.0861 $[C_8H_{11}]^+$, 121.1017 $[C_9H_{13}]^+$. Most sesquiterpenes produce fragments at *m/z* values of 147.1174 $[C_{11}H_{15}]^+$, 161.1330 $[C_{12}H_{17}]^+$, 175.1487$[C_{13}H_{19}]^+$ and 189.1643 $[C_{14}H_{21}]^+$.

Complementarily, some terpenoids present characteristic fragments that contain the ether oxygen or the hydroxyl group. Some examples of these fragments, shown in Supplementary Fig. 5, are found at *m/z* values 57.0334 $[C_3H_5O]^+$ for fenchol, 59.0497 $[C_3H_7O]^+$ for β-eudesmol, guaiol, α-terpineol and eucalyptol, 71.0497 $[C_4H_7O]^+$ for *trans*-phytol, menthol and α-terpineol, 85.0653 $[C_5H_9O]^+$ for menthol and α-terpineol, 111.0810 $[C_7H_{11}O]^+$ for eucalyptol and fenchol, 113.0966 $[C_7H_{13}O]^+$ for menthol, 125.0966 $[C_8H_{13}O]^+$ for eucalyptol and 139.1123 $[C_9H_{15}O]^+$ for α-terpineol, eucalyptol and fenchol, 205.1582 $[C_{14}H_{21}O]^+$ for caryophyllene oxide and 207.1749 $[C_{14}H_{23}O]^+$ for *cis*-nerolidol.

Fig. 2 clearly shows that monoterpenoids reported the highest intensity signals in hexanoic extracts, then sesquiterpenoids and, finally, tri- and diterpenoids. Among them, limonene (monocyclic monoterpene) was the terpenoid that provided the highest chromatographic peak, which can be explained by its role as precursor of monoterpenoids. Two caryophyllene isomers (the most common sesquiterpene [34]), β-myrcene and α-pinene were other terpenoids found at relatively high concentration.

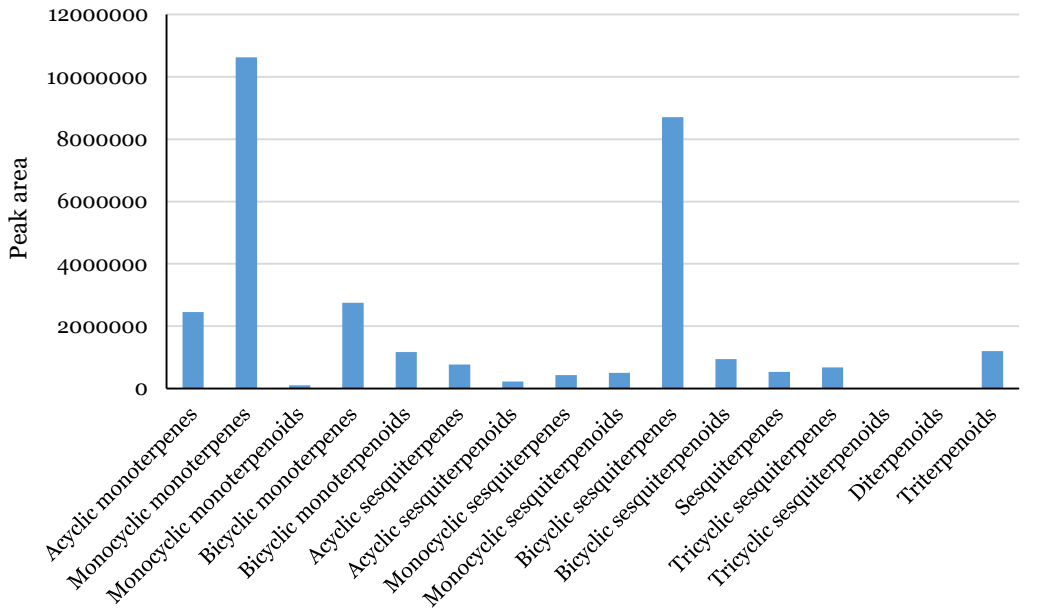

Fig. 2. Bars diagram comparing the peak areas of the identified terpenoids identified in *Cannabis* extracts by GC–TOF/MS, classified by subfamilies.

3.4. Other families of compounds identified in Cannabis

In addition to cannabinoids and terpenoids, other chemical families of compounds were tentatively identified in *Cannabis*. They included lipids, flavonoids, amino acids, organic acids, benzenoids, organic oxygen compounds, carbohydrates, hydrocarbons, organoheterocyclic compounds, organonitrogen compounds and an alkaloid.

Fig. 3 plots the Venn diagram that compares the number of metabolites identified using each of the two analytical platforms involved in this study. A total of 169 metabolites were detected by combination of the two platforms, with GC–MS reporting the highest detection coverage (~80% of the identified compounds). GC–MS was preferred to identify terpenoids, cannabinoids, lipids, benzene derivatives, hydrocarbons, organic acids and organic oxygen compounds; while LC–MS/MS allowed identification of flavonoids and amino acids, but it was also capable of covering compounds such as cannabinoids, carbohydrates and lipids, among others.

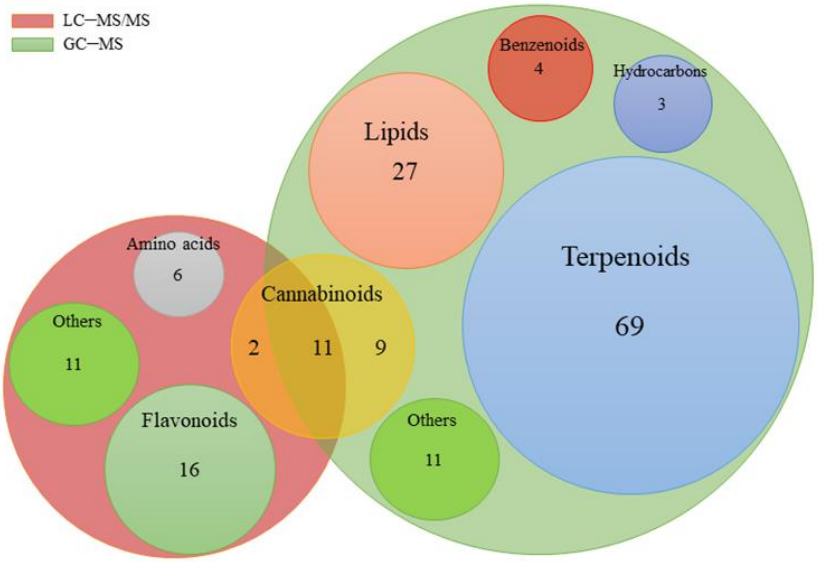

Fig. 3. Venn diagram comparing the number of compounds identified in *Cannabis* extracts by GC–TOF/MS and LC–QTOF MS/MS, classified by families.

Fig. 4 plots the bar diagram that compares the metabolites identified in *Cannabis* polar extracts using LC–MS/MS in negative and positive ionization modes, and also those identified in no polar extracts using GC–MS with and without previous derivatization. Some families such as lipids, flavonoids and carbohydrates provided a better response in negative ionization mode, while others as amino acids were better detected in positive mode, and cannabinoids in both ionization modes. Regarding families detected by GC–MS, lipids and organic acids provided a better response after derivatization of the extracts, while cannabinoids, terpenoids, benzenoids, organic oxygen compounds and hydrocarbons were better detected by direct analysis of the extracts.

Lipids are other key group of metabolites tentatively identified in *Cannabis*, representing 15% of all metabolites. They included mainly fatty acids, fatty acid derivatives such as hydroxy fatty acids, methyl-branched fatty acids, fatty acid esters, monoacylglycerols and steroids. Among them, more than 60% of identified lipids were fatty acids and derivatives. Fatty acids detected in *Cannabis* were mostly saturated, from medium to long-chains [caprylic acid, (C8:0), pelargonic acid (C9:0), lauric acid (C12:0), myristic acid (C14:0), pentadecylic acid (C15:0), palmitic (C16:0) margaric acid (C17:0),

stearic acid (C18:0) and arachidic acid (C20:0)]. Only five unsaturated fatty acids [oleic acid (C18:1 cis-9), its isomer, linoleic acid (C18:2), pinolenic (C18:3) and α-linolenic (C18:3) acids] were found. Some of them, such as oleic, arachidic and stearic acids, had been previously identified as the most abundant fatty acids in *Cannabis* seeds oil [34,35].

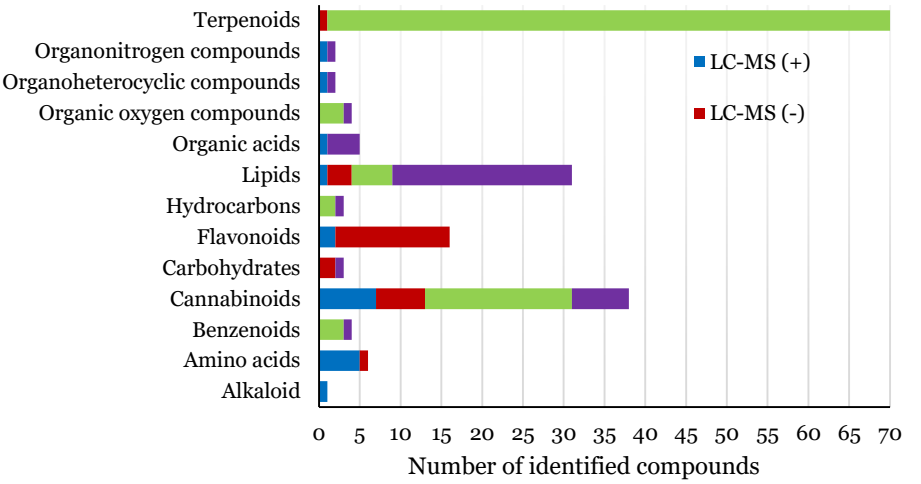

Fig. 4. Bars diagram comparing the number of compounds identified in *Cannabis* extracts by GC–TOF/MS, GC–TOF/MS after derivatization, LC–QTOF MS/MS in positive and negative ionization modes, classified by families.

Medium-chain dicarboxylic acids such as adipic and azelaic acids were also identified. Concerning fatty acid derivatives, 2 methyl-branched fatty acids (methylsuccinic and methylmaleic acids), a hydroxy fatty acid (ricinoleic acid), three fatty acid esters, two methyl esters from C16:0 and C18:0 and mono(2-ethylhexyl) adipate, and the fatty acid amide from C18:1 (oleamide) were also found in no polar extracts. Finally, other important compounds such as two steroids (4,14-dimethyl-9,19-cycloergost-24(28)-en-3-ol and 17β-hydroxyandrost-4-ene-3,11-dione propionate), tocopherol (vitamin E) and glycerophosphocholine (GPC) were reported.

Flavonoids are other key group of compounds identified in *Cannabis*. About 20 flavonoids have been reported to be present in the *Cannabis* plant as aglycons or as conjugated O-glycosides or C-glycosides [36–39]. The exact structure of most of the glycosides has not been determined because of the uncertainty of the exact linkage and/or

the number of sugar moieties. Paris *et al.* reported the detection of two flavonoids in the extracts of pollen grains from *Cannabis sativa*, which provided, upon acid hydrolysis, two genins identified as apigenin and luteolin, indicating the presence of the respective O-glycosides in the pollen [38].

Typical compounds such as quercetin and luteolin, and two of its glycosylated derivatives (quercetin-3-galactoside and homoorientin), two glycosylated derivatives of kaempferol (kaempferol-7-O-glucoside and kaempferol-rutinoside), one O-methylated flavone (diosmetin), one trihydroxyflavone (apigenin) and three of its glycosylated derivatives (vitexin, apigenin-7-O-glucoside and vitexin-2-rhamnoside), other glycosylated flavone (baicalin) and four flavanols (two procyanidins B and two procyanidins C) were tentatively identified. Some of them, as vitexin, were previously reported in low-THC *Cannabis* cultivars [34].

Flavonoids fragmentation patterns are very similar. Nevertheless, identification is possible by comparison of their retention times with those of the corresponding standards. For this reason, most flavonoids were identified injecting their individual standards, and the rest were tentatively identified by the commented libraries.

Amino acids were identified in the methanolic extracts of *Cannabis* using LC–QTOF MS/MS. Two essential amino acids (phenylalanine and tryptophan), two conditionally essential amino acids (arginine and proline), one no essential amino acid (glutamic acid) and one no proteinogenic amino acid (betaine) were found.

Metabolites of vitamins from the B complex (choline and trigonelline, previously identified in *Cannabis* [34]) and 4-formyl indole were tentatively identified in positive ionization mode by LC–QTOF MS/MS, while ethylamine and 2,5-dimethylimidazole were tentatively identified by GC–TOF/MS in the derivatized extracts. Ethylamine had been also previously identified in *Cannabis* extracts [34].

3.5. Comparison of cannabinoids and terpenoids in plants grown in greenhouse vs. field cultivation

Cannabis plants can exhibit wide variation in the quantity and type of cannabinoids and terpenoids they produce depending on the cultivar and growing conditions. The cultivation of plants in greenhouse (semiclosed environment) or in field

can influence many biochemical processes, including those involved in the formation of cannabinoids and terpenoids.

The cannabinoid and terpenoid profiles of female flowers collected from four different cultivars ('Theresa', 'Pilar', 'Aida' and 'Juani') grown in field in 2016 were compared with those provided by the same cultivars in greenhouse (Fig. 5 and Supplementary Fig. 6). The growing conditions (greenhouse or field) affected more significantly to cannabinoids as compared to terpenoids in all four cultivars.

Hexane extracts from 'Pilar' and 'Theresa' cultivars grown in greenhouse (Fig. 5A and C) and field (Fig. 5B and D) presented similar relative content of cannabinoids with predominance of CBD (>90% and >70%, respectively). Then, particular differences were found for each cultivar, which could be explained by the cultivation type. Thus, the content of CBG, CBC, cannabinerol and an isomer of Δ9-THCV in 'Pilar' plants cultivated in greenhouse was higher (0.49%, 4.41% 0.04% and 0.0029%, respectively) than in plants cultivated in field (0.09%, 2.01% 0.00% and 0.0002%, respectively). On the other hand, the content of CBGV, CBN, CBDV and an isomer of CBC in plants cultivated in greenhouse (0.07%, 0.16%, 0.30% and 0.06, respectively) was lower than in field (0.16%, 0.32%, 0.51% and 0.16%, respectively).

Concerning 'Theresa' cultivar, CBC, CBDV and Δ9-THC were high concentrated in both types of cultivation. The main difference found in the two extracts affected to Δ9-THCV, CBDV and an isomer of CBC that were found in plants grown in greenhouse at lower concentration (0.19%, 17.22% and 0.06%, respectively) than in field (0.26%, 21.56% and 0.08%, respectively).

'Aida' and 'Juani' cultivars grown in greenhouse (Fig. 5E and G) and field (Fig. 5F and H) were characterized by high content of CBG (>55% and >60%, respectively). Other dominant cannabinoids in the two cultivars were CBD, CBC and cannabinerol. The main difference between the two types of cultivation for 'Aida' cultivar affected to one isomer of CBC, found at higher concentration in plants cultivated in greenhouse (0.28%) as compared to those cultivated in field (0.11%).

Concerning CBGV, CBL, CBDV and Δ9-THC, they were at higher level in 'Aida' plants cultivated in field (0.23%, 0.51%, 0.40% and 0.43%, respectively) as compared to those grown in greenhouse (0.11%, 0.00%, 0.20% and 0.28%, respectively).

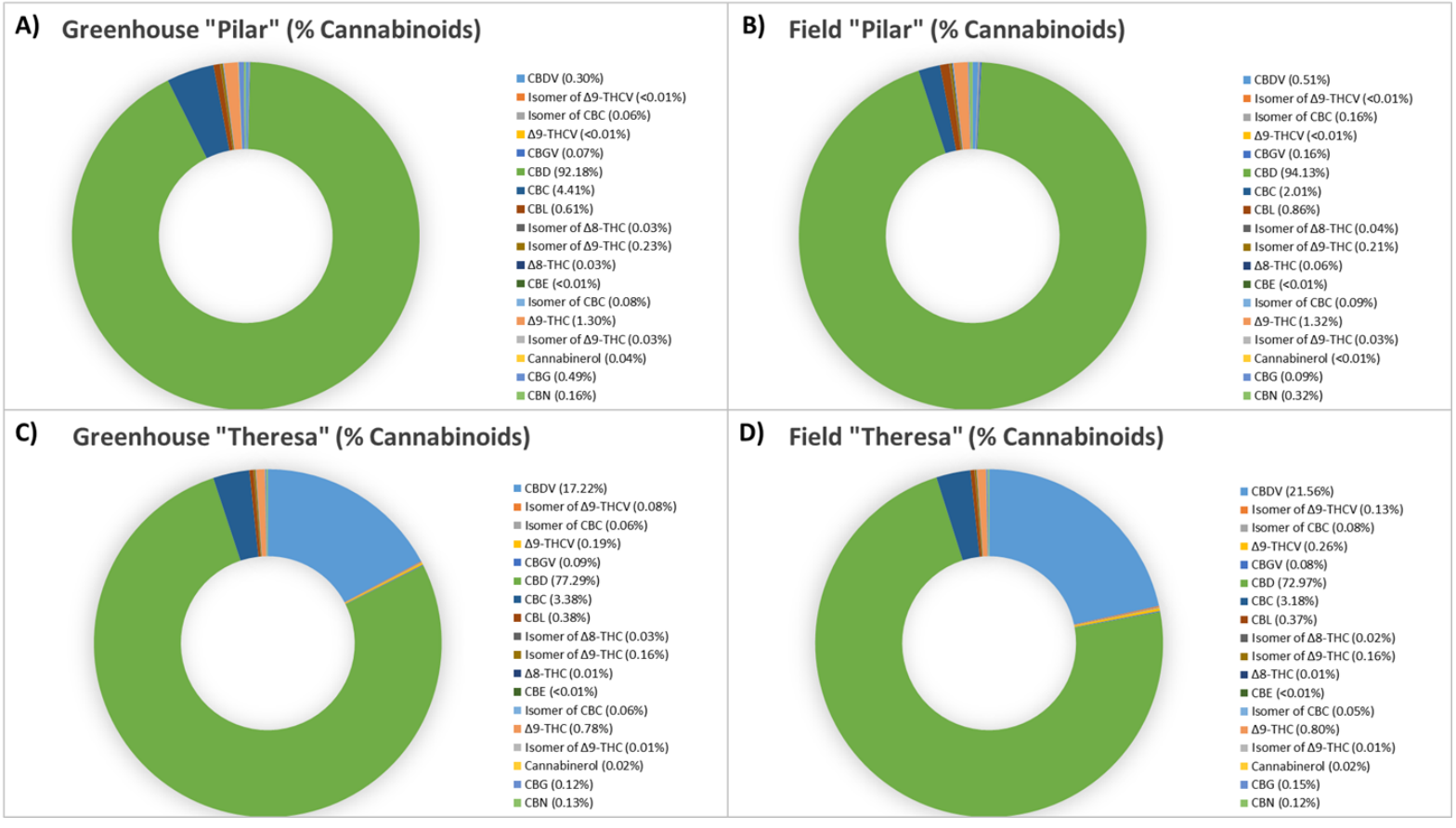

Fig. 5. Ring plots comparing the peak area of cannabinoids identified in extracts from two varieties ['Pilar' (**A** and **B**), and 'Theresa' (**C** and **D**)] of female *Cannabis* flowers, all cultivated in either field and or in greenhouse.

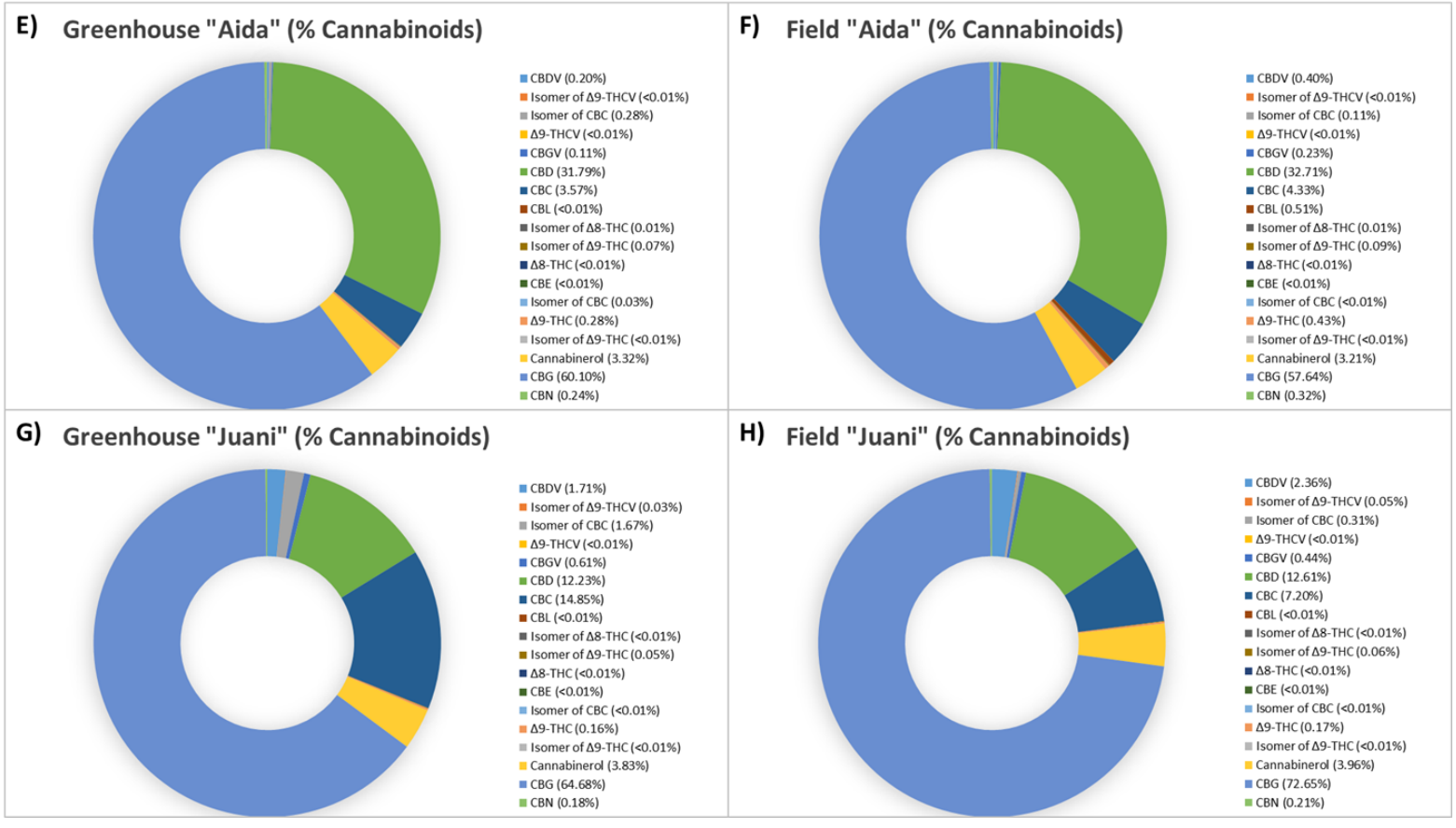

Fig. 5. Ring plots comparing the peak area of cannabinoids identified in extracts from two varieties [‘Aida’ (**E** and **F**), and ‘Juani’ (**G** and **H**)] of female *Cannabis* flowers, all cultivated in either field and or in greenhouse.

In the case of 'Juani' cultivar, the content of CBGV and one isomer of CBC in plants grown in greenhouse (0.61% and 1.67%, respectively) was higher than in plants cultivated in field (0.44% and 0.31%, respectively).

The terpenoids profile was also compared in the same four cultivars grown in greenhouse and field. High concentration of limonene was found in 'Pilar' (>70%) and 'Juani' (>30%) extracts. Limonene, α-pinene, β-myrcene, (-)-β-pinene, *trans*-caryophyllene and borneol were among the most concentrated terpenoids in all studied cultivars.

In general, the content of terpenoids was higher in extracts from plants grown in greenhouse than those cultivated in field. However, the content of caryophyllene and bergamotene isomers, caryophyllene oxide, squalene and β-amyrin was lower in cultivars grown in greenhouse. The content of α-pinene was higher in "Juani" cultivar grown in field. Other minor terpenoids such as β-myrcene, cedrane isomers, farnesene, α-curcumene, *cis*-thujopsene, β-guaiene, camphene, valencene, aristolene and selinene isomers were more concentrated in cultivars grown in field.

4. Conclusions

Untargeted metabolomics analysis of polar and no polar extracts from 21 *Cannabis sativa* L. cultivars has been carried out. Two analytical platforms, GC–TOF/MS and LC–QTOF MS/MS, were used to maximize the detection coverage. The first one was used to analyze the polar extracts and the second for no polar extracts, either with or without previous silylation of the target compounds. Forty-six compounds were identified in polar extracts and 134 compounds in no polar extracts, being 11 cannabinoids identified by both platforms. Among the identified families, mainly cannabinoids, terpenoids, lipids and flavonoids, but also some interesting compounds such as amino and organic acids as the most remarkable were found. Compositional differences were found in the profile of terpenoids and cannabinoids between extracts of plants cultivated in field and greenhouse.

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Supplementary Table 1. Identified compounds in no polar extracts from *Cannabis* analyzed by GC–TOF/MS with previous derivatization, and the main parameters that support their identification.

Family	Compound name	Formula	Mass	RT (min)	CAS ID	Compound name (derivatized product)	Mass (derivatized product)	Formula (derivatized product)	RI difference*	Fragments (derivatized product)	Identification
Benzenoid	1-Ethyl-2-methylbenzene	C9H12	120.0940	10.84	611-14-3	Benzene, 1-ethyl-2-methyl-	120.0940	C9H12	-96	105.0710 – $[C8H9]^+$ 103.0553 – $[C8H7]^+$ 91.0552 – $[C7H7]^+$	Tentative identification**
Cannabinoids	CBDV	C19H26O2	286.1927	44.63	24274-48-4	CBDV diTMS	430.2718	C25H42O2Si2		362.2062 – $[C20H34O2Si2]^+$ 273.1653 – $[C17H25OSi]^+$ 207.0321 – $[C9H11O2Si2]^+$	Standard
Cannabinoids	CBD	C21H30O2	314.2240	46.25	521-37-9	CBD diTMS	458.3031	C27H46O2Si2		390.2403 – $[C26H34OSi]^+$ 337.2007 – $[C22H29OSi]^+$ 301.1977 – $[C19H29OSi]^+$	Standard
Cannabinoids	CBL	C21H30O2	314.2240	47.25	21366-37-0	CBL TMS	386.2636	C24H38O2Si		303.1754 – $[C18H27O2Si]^+$ 281.0511 207.0327	Standard
Cannabinoids	CBDVA	C20H26O4	330.1826	47.76	31932-13-5	CBDVA triTMS	546.3011	C29H50O4Si3		463.2151 – $[C23H39O4Si3]^+$ 281.0505 – $[C11H17O3Si3]^+$ 207.0320 – $[C5H15O3Si3]^+$	Standard
Cannabinoids	Δ9-THC	C21H30O2	314.2240	47.98	1972-08-3	Δ9-THC TMS	386.2634	C24H38O2Si		371.2405 – $[C23H35O2Si]^+$ 315.1780 – $[C19H27O2Si]^+$ 303.1778 – $[C18H27O2Si]^+$	Standard
Cannabinoids	CBN	C21H26O2	310.1927	49.32	521-35-7	CBN TMS	382.2321	C24H34O2Si		367.2093 – $[C23H31O2Si]^+$ 310.1391 – $[C19H22O2Si]^+$ 207.0327	Standard
Cannabinoids	THCA	C22H30O4	358.2139	53.23	23978-85-0	THCA diTMS	502.2904	C28H46O4Si2		487.2707 – $[C27H43O4Si2]^+$ 419.2063 – $[C22H35O4Si2]^+$ 365.1592 – $[C18H29O4Si2]^+$	Standard
Carbohydrate (sugar alcohol)	Glycerol	C3H8O3	92.0468	27.33	56-81-5	Glycerol, tris(trimethylsilyl) ether	308.1659	C12H32O3Si3	0	205.1079 – $[C8H21O2Si2]^+$ 117.0367 – $[C4H9O2Si]^+$ 103.0571 – $[C4H11OSi]^+$	Tentative identification**
Hydrocarbon	1-Hexadecyne	C16H30	222.2331	44.10	629-74-3	1-Hexadecyne	222.2331	C16H30	96	95.0854 – $[C7H11]^+$ 81.0700 – $[C6H9]^+$ 67.0543 – $[C5H7]^+$	Tentative identification**
Lipids (fatty acid methyl ester)	Methyl stearate	C19H38O2	298.2872	45.01	112-61-8	Methyl stearate	298.2872	C19H38O2	23	199.1693 – $[C12H23O2]^+$ 143.1070 – $[C8H15O2]^+$ 87.0447 – $[C4H7O2]^+$	Tentative identification**
Lipids (fatty acid ester)	Mono(2-ethylhexyl) adipate	C14H26O4	258.1839	48.15	4337-65-9	Hexanedioic acid, mono(2-ethylhexyl)ester	258.1839	C14H26O4	98	129.0542 – $[C6H9O3]^+$ 111.0438 – $[C6H7O2]^+$ 101.0592 – $[C5H9O2]^+$	Tentative identification**
Lipids (fatty amide)	Oleamide	C18H35NO	281.2713	48.52	301-02-0	Oleamide, N-trimethylsilyl-	353.3114	C21H43NOSi	-27	338.2887 – $[C20H40NOSi]^+$ 128.0531 – $[C5H10NOSi]^+$ 116.0526 – $[C4H10NOSi]^+$	Tentative identification**
Lipids (glycerolipids)	1-Monomyristin	C17H34O4	302.2452	48.16	589-68-4	Myristic acid, 2,3-bis(trimethylsiloxy)-propyl ester	446.3248	C23H50O4Si2	-94	343.2678 – $[C19H39O3Si]^+$ 211.2067 – $[C14H27O]^+$ 129.0696 – $[C10H9]^+$	Tentative identification**
Lipids (glycerolipids)	1-Monopalmitin	C19H38O4	330.2765	51.65	542-44-9	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]-propyl ester	474.3561	C25H54O4Si2	-31	371.3005 – $[C21H43O3Si]^+$ 239.2385 – $[C16H31O]^+$ 129.1014 – $[C10H9]^+$	Tentative identification**
Lipids (glycerolipids)	2-Monostearin	C21H42O4	358.3078	54.55	621-61-4	2-Monostearin trimethylsilyl ether	502.3874	C27H58O4Si2	-30	203.0921 – $[C8H19O2Si2]^+$ 129.0727 – $[C6H13OSi]^+$ 103.0572 – $[C4H11OSi]^+$	Tentative identification**
Lipids (glycerolipids)	1-Monostearin	C21H42O4	358.3078	54.88	123-94-4	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]-propyl ester	502.3874	C27H58O4Si2	94	399.3327 – $[C23H47O3Si]^+$ 203.0932 – $[C8H19O2Si2]^+$ 129.0723 – $[C6H13OSi]^+$	Tentative identification**
Lipids (long-chain fatty acids)	Myristic acid (C14:0)	C14H28O2	228.2084	42.72	544-63-8	Tetradecanoic acid, trimethylsilyl ester	300.2485	C17H36O2Si	12	257.1012 – $[C15H17O2Si]^+$ 129.0377 – $[C5H9O2Si]^+$ 117.0373 – $[C4H9O2Si]^+$	Tentative identification
Lipids (long-chain fatty acids)	Pentadecylic acid (C15:0)	C15H30O2	242.2240	43.47	1002-84-2	n-Pentadecanoic acid, trimethylsilyl ester	314.2641	C18H38O2Si	12	299.2407 – $[C17H35O2Si]^+$ 129.0376 – $[C5H9O2Si]^+$ 117.0370 – $[C4H9O2Si]^+$	Tentative identification**

Continuation Supplementary Table 1

Family	Compound name	Formula	Mass	RT (min)	CAS ID	Compound name (derivatized product)	Mass (derivatized product)	Formula (derivatized product)	RI difference*	Fragments (derivatized product)	Identification
Lipids (long-chain fatty acids)	Palmitic acid (C16:0)	C16H32O2	256.2397	44.26	57-10-3	Hexadecanoic acid, trimethylsilyl ester	328.2772	C19H40O2Si	13	313.2584 – $[C18H37O2Si]^+$ 129.0376 – $[C5H9O2Si]^+$ 117.0375 – $[C4H9O2Si]^+$	Tentative identification
Lipids (long-chain fatty acids)	Margaric acid (C17:0)	C17H34O2	270.2553	44.87	506-12-7	Heptadecanoic acid, trimethylsilyl ester	342.2954	C20H42O2Si	13	327.2717 – $[C19H39O2Si]^+$ 129.0375 – $[C5H9O2Si]^+$ 117.0369 – $[C4H9O2Si]^+$	Tentative identification
Lipids (long-chain fatty acids)	Oleic acid (C18:1)	C18H34O2	282.2553	45.89	112-80-1	Oleic acid, trimethylsilyl ester	354.2968	C21H42O2Si	6	339.2726 – $[C20H39O2Si]^+$ 129.0368 – $[C5H9O2Si]^+$ 117.0368 – $[C4H9O2Si]^+$	Tentative identification
Lipids (long-chain fatty acids)	Isomer of oleic acid (C18:1)	C18H34O2	282.2553	45.96		Oleic acid, trimethylsilyl ester 1	354.2954	C21H42O2Si	6	339.2722 – $[C20H39O2Si]^+$ 129.0371 – $[C5H9O2Si]^+$ 117.0371 – $[C4H9O2Si]^+$	Not exactly defined compound
Lipids (long-chain fatty acids)	Stearic acid (C18:0)	C18H36O2	284.2710	46.19	57-11-4	Octadecanoic acid, trimethylsilyl ester	356.3081	C21H44O2Si	20	341.2894 – $[C20H41O2Si]^+$ 129.0373 – $[C5H9O2Si]^+$ 117.0373 – $[C4H9O2Si]^+$	Tentative identification
Lipids (long-chain fatty acids)	Arachidic acid (C20:0)	C20H40O2	312.3023	48.94	506-30-9	Eicosanoic acid, trimethylsilyl ester	384.3424	C23H48O2Si	15	369.3197 – $[C22H45O2Si]^+$ 129.0369 – $[C5H9O2Si]^+$ 117.0369 – $[C4H9O2Si]^+$	Tentative identification
Lipids (medium-chain fatty acids)	Caprylic acid (C8:0)	C8H16O2	144.1145	26.71	124-07-2	Octanoic acid, trimethylsilyl ester	216.1546	C11H24O2Si	40	201.1308 – $[C10H21O2Si]^+$ 129.0367 – $[C5H9O2Si]^+$ 117.0364 – $[C4H9O2Si]^+$	Tentative identification
Lipids (medium-chain fatty acids)	Pelargonic acid (C9:0)	C9H18O2	158.1301	30.48	112-05-0	Nonanoic acid, trimethylsilyl ester	230.1702	C12H26O2Si	-8	215.1472 – $[C11H23O2Si]^+$ 129.0374 – $[C5H9O2Si]^+$ 117.0370 – $[C4H9O2Si]^+$	Tentative identification
Lipids (medium-chain fatty acids)	Adipic acid	C6H10O4	146.0574	35.57	124-04-9	Hexanedioic acid, bis(trimethylsilyl) ester	290.1370	C12H26O4Si2	-25	185.0611 – $[C8H13O3Si]^+$ 141.0724 – $[C7H13OSi]^+$ 111.0436 – $[C6H7O2]^+$	Tentative identification**
Lipids (medium-chain fatty acids)	Lauric acid (C12:0)	C12H24O2	200.1776	40.43	143-07-7	Dodecanoic acid, trimethylsilyl ester	272.2166	C15H32O2Si	100	257.1934 – $[C14H29O2Si]^+$ 129.0377 – $[C5H9O2Si]^+$ 117.0376 – $[C4H9O2Si]^+$	Tentative identification**
Lipids (medium-chain fatty acids)	Azelaic acid	C9H16O4	188.1049	42.31	123-99-9	Azelaic acid, bis(trimethylsilyl) ester	332.1839	C15H32O4Si2	93	317.1606 – $[C14H29O4Si2]^+$ 201.1324 – $[C10H21O2Si]^+$ 117.0380 – $[C4H9O2Si]^+$	Tentative identification
Lipids (methyl-branched fatty acids)	Methyl succinic acid	C5H8O4	132.0417	29.85	3641-51-8	Butanedioic acid, methylene-, bis(trimethylsilyl) ester	274.1057	C11H22O4Si2	-50	259.0815 – $[C10H19O4Si2]^+$ 215.0914 – $[C9H19O2Si2]^+$ 97.0466 – $[C5H9Si]^+$	Tentative identification**
Lipids (methyl-branched fatty acids)	Methylmaleic acid	C5H6O4	130.0261	31.91	498-23-7	Methylmaleic acid, bis(trimethylsilyl) ester	274.1057	C11H22O4Si2	69	259.0820 – $[C10H19O4Si2]^+$ 133.0134 – $[C3H9O2Si2]^+$ 97.0454 – $[C5H9Si]^+$	Tentative identification**
Organic acids (carbamic acids)	Carbamate	CH3NO2	61.0158	11.90	463-77-4	Tris(trimethylsilyl)carbamate	277.1350	C10H27NO2Si3	-62	262.1112 – $[C9H24NO2Si3]^+$ 174.0403 – $[C5H12NO2Si2]^+$ 131.0343 – $[C4H11OSi2]^+$	Tentative identification**
Organic acids (carbamic acids)	N,N-Diethylcarbamate	C5H10NO2	116.0706	13.22	24579-70-2	N,N-Diethyl-(trimethylsilyl)carbamate	189.1185	C8H19NO2Si	7	174.0946 – $[C7H16NO2Si]^+$ 130.1044 – $[C6H16NSi]^+$ 100.0745 – $[C5H10NO]^+$	Tentative identification**
Organic acids (dicarboxylic acid)	Ethanedioic acid	C2H2O4	89.9948	13.59	144-62-7	Ethanedioic acid, bis(trimethylsilyl) ester	234.0744	C8H18O4Si2	-94	131.0336 – $[C5H7O4]^+$ 117.0727 – $[C5H13OSi]^+$ 73.0464 – $[C3H9Si]^+$	Tentative identification**
Organic acids (mercaptocarboxylic acid)	Mercaptoacetic acid	C2H4O2S	91.9927	11.38	68-11-1	Mercaptoacetic acid, bis(trimethylsilyl)-	236.0723	C8H20O2SSi2	-95	147.0681 – $[C6H15SSi]^+$ 115.0040 – $[C4H7SSi]^+$ 73.0479 – $[C3H9Si]^+$	Tentative identification**
Organic oxygen compound (ketone)	4-Hydroxy-3-penten-2-one	C5H8O2	100.0519	16.41		4-(Trimethylsilyoxy)-3-penten-2-one	172.0920	C8H16O2Si	77	157.0697 – $[C7H13O2Si]^+$ 115.0573 – $[C5H11OSi]^+$ 85.0463 – $[C4H9Si]^+$	Tentative identification**
Organoheterocyclic compound (imidazole)	2,5-Dimethylimidazole	C5H8N2	96.0682	26.19		Imidazole, 2,5-dimethyl-4-trifluoromethyl-	164.0563	C6H7F3N2	93	163.0486 – $[C6H6N2F3]^+$ 145.0573 – $[C6H7N2F2]^+$ 95.0614 – $[C5H7N2]^+$	Tentative identification**
Organonitrogen compound (amine)	Ethylamine	C2H7N	45.0573	7.85	75-04-7	Ethylbis(trimethylsilyl)amine	189.1354	C8H23NSi2	64	174.1140 – $[C7H20NSi2]^+$ 130.0507 – $[C4H12NSi2]^+$ 100.0581 – $[C4H10NSi]^+$	Tentative identification

*RI difference = Experimental RI - NIST RI; ** New compound identified in *Cannabis*.

Supplementary Table 2. Identified compounds in no polar extracts from *Cannabis* analyzed by GC–TOF/MS without previous derivatization and the main parameters that support their identification.

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Benzenoids	3,5-Dimethylbenzaldehyde	C9H10O	134.0719	23.04	-8	5779-95-3	105.0694 – $[C8H9]^+$ 91.0538 – $[C7H7]^+$ 77.0382 – $[C6H5]^+$	Tentative identification**
Benzenoids	1,3-Di-*tert*-butylbenzene	C14H22	190.1707	25.28	-84	1014-60-4	175.1479 – $[C13H19]^+$ 91.0536 – $[C7H7]^+$ 57.0697 – $[C4H9]^+$	Tentative identification**
Benzenoids	2,4-Di-*tert*-butylphenol	C14H22O	206.1659	35.51	-39	96-76-4	191.1430 – $[C13H19O]^+$ 163.1112 – $[C11H15O]^+$ 115.0534 –$[C9H7]^+$	Tentative identification**
Cannabinoids	CBDV	C19H26O2	286.1934	46.01		24274-48-4	203.1086 –$[C13H15O2]^+$ 174.0686 – $[C11H10O2]^+$ 218.1301 – $[C14H18O2]^+$ 271.1700 – $[C18H23O2]^+$	Standard
Cannabinoids	Isomer of Δ9-THCV	C19H26O2	286.1933	46.79	24		203.1073 – $[C13H15O2]^+$ 271.1687 – $[C18H23O2]^+$ 243.1376 – $[C16H19O2]^+$	Not exactly defined compound
Cannabinoids	Isomer of CBC	C21H30O2	314.2246	46.88	-95		231.1386 – $[C15H19O2]^+$ 174.0671 – $[C11H10O2]^+$ 271.1689 – $[C18H23O2]^+$	Not exactly defined compound
Cannabinoids	Δ9-THCV	C19H26O2	286.1933	47.23		31262-37-0	271.1711 – $[C18H23O2]^+$ 203.1082 – $[C13H15O2]^+$ 243.1395 – $[C16H19O2]^+$	Standard
Cannabinoids	CBGV	C19H28O2	288.2078	48.03	44	55824-11-8	165.0906 – $[C10H13O2]^+$ 231.1373 – $[C15H19O2]^+$ 203.1063 – $[C13H15O2]^+$ 123.1156 – $[C9H15]^+$	Tentative identification
Cannabinoids	CBD	C21H30O2	314.2241	48.75		13956-29-1	231.1391 – $[C15H19O2]^+$ 174.0676 – $[C11H10O2]^+$ 246.1613 – $[C16H22O2]^+$	Standard
Cannabinoids	CBC	C21H30O2	314.2235	48.89		20675-51-8	231.1391 – $[C15H19O2]^+$ 174.0678 – $[C11H10O2]^+$ 271.1691– $[C18H23O2]^+$	Standard
Cannabinoids	CBL	C21H30O2	314.2241	48.97	-86	21366-63-22	231.1416 – $[C15H19O2]^+$	Tentative identification
Cannabinoids	Isomer of Δ8-THC	C21H30O2	314.2231	49.60	25		231.1375 – $[C15H19O2]^+$ 258.1602 – $[C17H22O2]^+$ 193.1212 – $[C12H17O2]^+$ 271.1688 – $[C18H23O2]^+$	Not exactly defined compound
Cannabinoids	Isomer of Δ9-THC	C21H30O2	314.2235	49.82	25		231.1379 – $[C15H19O2]^+$ 299.2003 – $[C20H27O2]^+$ 271.1688 – $[C18H23O2]^+$	Not exactly defined compound
Cannabinoids	Δ8-THC	C21H30O2	314.2251	50.03		5957-75-5	231.1396 – $[C15H19O2]^+$ 258.1621 – $[C17H22O2]^+$ 271.1700 – $[C18H23O2]^+$	Standard
Cannabinoids	CBE	C21H30O3	330.2184	50.03	25	52025-76-0	231.1370 – $[C15H19O2]^+$ 205.1212 – $[C13H17O2]^+$ 247.1332 – $[C15H19O3]^+$	Tentative identification

Continuation Supplementary Table 2

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Cannabinoids	Isomer of CBC	C21H30O2	314.2243	50.15	14		231.1376 – $[C15H19O2]^+$ 193.1220 – $[C12H17O2]^+$ 174.0675 – $[C11H10O2]^+$	Not exactly defined compound
Cannabinoids	Δ9-THC	C21H30O2	314.2250	50.49		1972-08-3	299.2013 – $[C20H27O2]^+$ 231.1393 – $[C15H19O2]^+$ 271.1702 – $[C18H23O2]^+$	Standard
Cannabinoids	Isomer of Δ9-THC	C21H30O2	314.2238	51.04	75		299.1999 – $[C20H27O2]^+$ 231.1377 – $[C15H19O2]^+$ 271.1684 – $[C18H23O2]^+$	Not exactly defined compound
Cannabinoids	Cannabinerol	C21H32O2	316.2387	51.36	-60		231.1373 – $[C15H19O2]^+$ 193.1217 – $[C12H17O2]^+$ 149.0598 – $[C9H9O2]^+$	Tentative identification
Cannabinoids	CBG	C21H32O2	316.2402	51.56		2808-33-5	193.1238 – $[C12H17O2]^+$ 231.1385 – $[C15H19O2]^+$ 123.1161 – $[C9H15]^+$	Standard
Cannabinoids	CBN	C21H26O2	310.1926	51.68		521-35-7	295.1707 – $[C20H23O2]^+$ 238.0990 – $[C16H14O2]^+$ 193.1217 – $[C12H17O2]^+$	Standard
Hydrocarbons	Heptacosane	C27H56	380.4382	56.72	95	593-49-7	85.1007 – $[C6H13]^+$ 71.0853 – $[C5H11]^+$ 57.0698 – $[C4H9]^+$	Tentative identification
Hydrocarbons	1,3,5-Cycloheptatriene	C7H8	92.0617	3.25	-96	544-25-2	89.0375 – $[C7H5]^+$ 65.0383 – $[C5H5]^+$ 63.0233 – $[C5H3]^+$	Tentative identification**
Lipids (fatty acid methyl ester)	Methyl palmitate	C17H34O2	270.2569	43.33	8	112-39-0	171.1373 – $[C10H19O2]^+$ 143.1058 – $[C8H15O2]^+$ 87.0436 – $[C4H7O2]^+$	Tentative identification
Lipids (long-chain fatty acids)	Linoleic acid	C18H32O2	280.2394	45.07	-83	60-33-3	67.0541 – $[C5H7]^+$ 81.0697 – $[C6H9]^+$ 95.0851 – $[C7H11]^+$	Tentative identification
Lipids (long-chain fatty acids)	α-Linolenic acid	C18H30O2	278.2233	45.13	-91	463-40-1	79.0545 – $[C6H7]^+$ 67.0542 – $[C5H7]^+$ 93.0697 – $[C7H9]^+$	Tentative identification
Lipids (prenol lipid)	Vitamin E	C29H50O2	430.3799	57.95	-99	10191-41-0	387.3245 – $[C24H43O2]^+$ 205.1214 – $[C13H17O2]^+$ 165.0904 – $[C10H13O2]^+$	Tentative identification**
Lipids (steroid)	4,14-Dimethyl-, (3β,4α,5α)-9,19-cycloergost-24(28)-en-3-ol	C30H50O	426.3820	62.34	100	469-39-6	408.3735 – $[C30H48]^+$ 393.3504 – $[C29H45]^+$ 353.3176 – $[C26H41]^+$	Tentative identification**
Organic oxygen compounds (aldehyde)	2-Ethylbutanal	C6H12O	100.0878	3.46	-92	97-96-1	71.0490 – $[C4H7O]^+$ 72.0567 – $[C4H8O]^+$ 85.0644 – $[C5H9O]^+$	Tentative identification**
Organic oxygen compounds (ketone)	2-Hexanone	C6H12O	100.0878	3.53	-94	591-78-6	58.0406 – $[C3H6O]^+$ 85.0640 – $[C5H9O]^+$ 71.0841 – $[C5H11]^+$	Tentative identification**
Organic oxygen compounds (aldehyde)	9,17-Octadecadienal	C18H32O	264.2434	44.78	-3	56554-35-9	67.0538 – $[C5H7]^+$ 81.0693 – $[C6H9]^+$ 95.0849 – $[C7H11]^+$	Tentative identification**
Terpenoids (monoterpenoids, bicyclic monoterpenes)	α-Pinene	C10H16	136.1246	7.50		80-56-8	91.0546 – $[C7H7]^+$ 77.0387 – $[C6H5]^+$ 105.0696 – $[C8H9]^+$	Standard
Terpenoids (monoterpenoids, bicyclic monoterpenes)	(-)-Camphene	C10H16	136.1243	8.16		79-92-5	93.0705 – $[C7H9]^+$ 121.1017 – $[C9H13]^+$ 79.0546 – $[C6H7]^+$	Standard
Terpenoids (monoterpenoids, bicyclic monoterpenes)	(-)-β-Pinene	C10H16	136.1243	9.35		18172-67-3	93.0704 – $[C7H9]^+$ 77.0388 – $[C6H5]^+$ 121.1010 – $[C9H13]^+$	Standard

Continuation Supplementary Table 2

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Terpenoids (monoterpenoids, acyclic monoterpenes)	β-Myrcene	C10H16	136.1241	9.94		123-35-3	93.0703 – $[C7H9]^+$ 77.0386 – $[C6H5]^+$ 69.0697 – $[C5H9]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenes)	α-Phellandrene	C10H16	136.1246	10.73		99-83-2	91.0550 – $[C7H7]^+$ 93.0705 – $[C7H9]^+$ 77.0392 – $[C6H5]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenes)	β-Terpinene	C10H16	136.1239	11.27	-93	99-86-5	121.1010 – $[C9H13]^+$ 93.0703 – $[C7H9]^+$ 77.0387 – $[C6H5]^+$	Tentative identification
Terpenoids (monoterpenoids, monocyclic monoterpenes)	*p*-Cymene	C10H14	134.1091	11.67		99-87-6	119.0864 – $[C9H11]^+$ 91.0549 – $[C7H7]^+$ 103.0541 – $[C8H7]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenes)	Limonene	C10H16	136.1242	11.90		5989-27-5	67.0546 – $[C5H7]^+$ 93.0701 – $[C7H9]^+$ 79.0544 – $[C6H7]^+$	Standard
Terpenoids (monoterpenoids, bicyclic monoterpenoids)	Eucalyptol	C10H18O	154.1350	12.04		470-82-6	93.0703 – $[C7H9]^+$ 81.0702 – $[C6H9]^+$ 139.1118 –6 $[C9H15O]^+$	Standard
Terpenoids (monoterpenoids, acyclic monoterpenes)	*trans*-β-Ocimene	C10H16	136.1241	12.34		3779-61-1	91.0544 – $[C7H7]^+$ 77.0386 – $[C6H5]^+$ 105.0697 – $[C8H9]^+$	Standard
Terpenoids (monoterpenoids, acyclic monoterpenes)	β-Ocimene	C10H16	136.1241	12.91			91.0546 – $[C7H7]^+$ 77.0387 – $[C6H5]^+$ 105.0698 – $[C8H9]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenes)	γ-Terpinene	C10H16	136.1244	13.50		99-85-4	91.0547 – $[C7H7]^+$ 77.0387 – $[C6H5]^+$ 121.1011 – $[C9H13]^+$	Standard
Terpenoids (monoterpenoids, bicyclic monoterpenoids)	(+)-Fenchol	C10H18O	154.1358	17.05		2217-02-9	81.0700 – $[C6H9]^+$ 93.0696 – $[C7H9]^+$ 111.0802 – $[C7H11O]^+$	Standard
Terpenoids (monoterpenoids, bicyclic monoterpenoids)	(-)-Borneol	C10H18O	154.1358	20.31		464-45-9	95.0860 – $[C7H11]^+$ 67.0541 – $[C5H7]^+$ 121.1009 – $[C9H13]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenoids)	Menthol	C10H20O	156.1514	20.78		2216-51-5	81.0702 – $[C6H9]^+$ 67.0544– $[C5H7]^+$ 95.0857 – $[C7H11]^+$	Standard
Terpenoids (monoterpenoids, monocyclic monoterpenoids)	α-Terpineol	C10H18O	154.1358	21.87		98-55-5	93.0702 – $[C7H9]^+$ 121.1014 – $[C9H13]^+$ 81.0699 – $[C6H9]^+$ 59.0491 – $[C3H7O]^+$	Standard
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenes)	β-Elemene	C15H24	204.1864	31.33		515-13-9	93.0702 – $[C7H9]^+$ 79.0545 – $[C6H7]^+$ 105.0701– $[C8H9]^+$	Standard
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (I)	C15H24		31.83			57.0694 – $[C4H9]^+$ 91.0535 – $[C7H7]^+$ 119.0846 – $[C9H11]^+$	Not defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (II)	C15H24	204.1860	32.00			79.0534 – $[C6H7]^+$ 105.0691 – $[C8H8]^+$ 147.1154 – $[C11H15]^+$	Not defined compound
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Cedrene	C15H24	204.1869	32.15		469-61-4	119.0861 – $[C9H11]^+$ 105.0700 – $[C8H9]^+$ 91.0542 – $[C7H7]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	*trans*-(-)-Caryophyllene	C15H24	204.1878	32.28		87-44-5	91.0546 – $[C7H7]^+$ 105.0701 – $[C8H9]^+$ 79.0543 – $[C6H7]^+$	Standard

Continuation Supplementary Table 2

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	*trans*-α-Bergamotene	C15H24	204.1866	32.79	-30	13474-59-4	119.0857 – $[C_9H_{11}]^+$ 93.0698 – $[C_7H_9]^+$ 91.0543 – $[C_7H_7]^+$	Tentative identification
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	Aromadendrene	C15H24	204.1867	32.90		489-39-4	91.0546 – $[C_7H_7]^+$ 105.0702 – $[C_8H_9]^+$ 79.0543 – $[C_6H_7]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	*cis*-(-)-2,4α,5,6,9α-Hexahydro-3,5,5,9-tetramethyl(1H)benzo-cycloheptene	C15H24	204.1871	33.28	-39		91.0534 – $[C_7H_7]^+$ 79.0536 – $[C_6H_7]^+$ 105.690 – $[C_8H_9]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Isomer of caryophyllene (I)	C15H24	204.1867	33.50	-94		93.0701 – $[C_7H_9]^+$ 77.0387 – $[C_6H_5]^+$ 105.0698 – $[C_8H_9]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (III)	C15H24	204.1856	33.62			91.0536 – $[C_7H_7]^+$ 57.0696 – $[C_4H_9]^+$ 71.0850 – $[C_5H_{11}]^+$	Not defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (IV)	C15H24		33.79			105.0690 – $[C_8H_9]^+$ 119.0845 – $[C_9H_{11}]^+$ 93.0688 – $[C_7H_9]^+$	Not defined compound
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenes)	β-Curcumene	C15H24	204.1861	34.38	-30	451-56-9	119.0848 – $[C_9H_{11}]^+$ 91.0536 – $[C_7H_7]^+$ 105.0691 – $[C_8H_9]^+$	Tentative identification
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenes)	α-Curcumene	C15H22	202.1702	34.51	-22	4176-17-4	132.0925 – $[C_{10}H_{12}]^+$ 119.0848 – $[C_9H_{11}]^+$ 105.0693 – $[C_8H_9]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Isomer or caryophyllene (II)	C15H24	204.1856	34.59	26		95.0846 – $[C_7H_{11}]^+$ 71.0851 – $[C_5H_{11}]^+$ 105.0693 – $[C_8H_9]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	β-Selinene	C15H24	204.1861	34.73	-19	17066-67-0	105.0691 – $[C_8H_9]^+$ 91.0535 – $[C_7H_7]^+$ 79.0536 – $[C_6H_7]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Valencene	C15H24	204.1870	34.88		4630-07-3	91.0546 – $[C_7H_7]^+$ 105.0703 – $[C_8H_9]^+$ 119.0858 – $[C_9H_{11}]^+$	Standard
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	Di-epi-α-cedrene	C15H24	204.1858	35.05	97		119.0848 – $[C_9H_{11}]^+$ 91.0537 – $[C_7H_7]^+$ 77.0380 – $[C_6H_5]^+$	Tentative identification
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenoids)	Dihydro-β-agarofuran	C15H26O		35.35	-14	5956-09-2	57.0695 – $[C_4H_9]^+$ 71.0850 – $[C_5H_{11}]^+$ 85.1005 – $[C_6H_{13}]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	*cis*-α-Bergamotene	C15H24	204.1864	35.61	70	18252-46-5	119.0851 – $[C_9H_{11}]^+$ 91.0538 – $[C_7H_7]^+$ 105.0693 – $[C_8H_9]^+$	Tentative identification
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (V)	C15H24	204.1863	35.74			95.0849 – $[C_7H_{11}]^+$ 67.0538 – $[C_5H_7]^+$ 109.1003 – $[C_8H_{13}]^+$	Not defined compound
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	Isomer of cedrene	C15H24	204.1861	35.87	85		69.0694 – $[C_5H_9]^+$ 105.0691 – $[C_8H_9]^+$ 119.0846 – $[C_9H_{11}]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenes)	β-Sesquiphellandrene	C15H24	204.1865	36.13	-16	20307-83-9	91.0537 – $[C_7H_7]^+$ 105.0692 – $[C_8H_9]^+$ 161.1318 – $[C_{12}H_{17}]^+$	Tentative identification
Terpenoids (sesquiterpenoids, acyclic sesquiterpenoids)	*cis*-Nerolidol	C15H26O	222.1984	36.40		7212-44-4	93.0702 – $[C_7H_9]^+$ 107.0857 – $[C_8H_{11}]^+$ 79.0543 – $[C_6H_7]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	β-Guaiene	C15H24	204.1864	36.54	-15	88-84-6	105.0693 – $[C_8H_9]^+$ 161.1316 – $[C_{12}H_{17}]^+$ 133.1002 – $[C_{10}H_{13}]^+$	Tentative identification

Continuation Supplementary Table 2

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Terpenoids (sesquiterpenoids, acyclic sesquiterpenes)	Farnesene	C15H24	204.1861	36.75		502-61-4	93.0697 – $[C_7H_9]^+$ 119.0851 – $[C_9H_{11}]^+$ 79.0539 – $[C_6H_7]^+$	Standard
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenoids)	4,6,6-Trimethyl-2-(3-methy-lbuta-1,3-dienyl)-3-oxatricyclo-[5.1.0.0(2,4)]octane	C15H22O	218.1659	37.02	93		105.0689 – $[C_8H_9]^+$ 91.0535 – $[C_7H_7]^+$ 133.0990 – $[C_{10}H_{13}]^+$	Tentative identification
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenes)	α-Humulene	C15H24	204.1860	37.51	58	6753-98-6	93.0692 – $[C_7H_9]^+$ 107.0847 – $[C_8H_{11}]^+$ 121.1002 – $[C_9H_{13}]^+$	Tentative identification
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	*cis*-Thujopsene	C15H24	204.1865	37.93	95	470-40-6	161.1316 – $[C_{12}H_{17}]^+$ 81.0691 – $[C_6H_9]^+$ 93.0689 – $[C_7H_9]^+$	Tentative identification
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenoids)	Caryophyllene oxide	C15H24O	220.1827	38.13		1139-30-6	91.0546 – $[C_7H_7]^+$ 79.0545 – $[C_6H_7]^+$ 105.0698 – $[C_8H_9]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenoids)	Guaiol	C15H26O		38.68		489-86-1	105.0704 – $[C_8H_9]^+$ 161.1311 – $[C_{12}H_{17}]^+$ 91.0543 – $[C_7H_7]^+$	Standard
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (VI)	C15H24	204.1858	38.91			57.0696 – $[C_4H_9]^+$ 107.0847 – $[C_8H_{11}]^+$ 71.0848 – $[C_5H_{11}]^+$	Not defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (VII)	C15H24	204.1865	39.09			67.0538 – $[C_5H_7]$+ 79.0536 – $[C_6H_7]$+ 161.1318 – $[C_{12}H_{17}]$+	Not defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (VIII)	C15H24	204.1856	39.22			149.1314 – $[C_{11}H_{17}]^+$ 93.0691 – $[C_7H_9]^+$ 108.0921 – $[C_8H_{12}]^+$	Not defined compound
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	4-Methylene-2,8,8-trimethyl-2-vinylbicyclo[5.2.0]nonane	C15H24	204.1856	39.33	93		67.0537 – $[C_5H_7]^+$ 81.0693 – $[C_6H_9]^+$ 55.0540 – $[C_4H_7]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	γ–Selinene	C15H24	204.1871	39.51	100	515-17-3	161.1325 – $[C_{12}H_{17}]^+$ 189.1628 – $[C_{14}H_{21}]^+$ 133.1010 – $[C_{10}H_{13}]^+$	Tentative identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Isomer of guaia-1(10),11-diene (I)	C15H24	204.1865	39.84	96		189.1632 – $[C_{14}H_{21}]^+$ 161.1320 – $[C_{12}H_{17}]^+$ 107.0848 – $[C_8H_{11}]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Isomer of guaia-1(10),11-diene (II)	C15H24	204.1863	39.96	96		119.0848 – $[C_9H_{11}]^+$ 161.1317 – $[C_{12}H_{17}]^+$ 107.0847 – $[C_8H_{11}]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	Isomer of longipinene	C15H24	204.1859	40.06	96		119.0847 – $[C_9H_{11}]^+$ 161.1318 – $[C_{12}H_{17}]^+$ 91.0535 – $[C_7H_7]^+$	Not exactly defined compound
Terpenoids (sesquiterpenoids, sesquiterpenes)	Sesquiterpene (IX)	C15H24	204.1868	40.16			81.0693 – $[C_6H_9]^+$ 107.0849 – $[C_8H_{11}]^+$ 93.0693 – $[C_7H_9]^+$	Not defined compound
Terpenoids (sesquiterpenoids, tricyclic sesquiterpenes)	(-)-Aristolene	C15H24	204.1866	40.24	99	6831-16-9	105.0694 – $[C_8H_9]^+$ 91.0538 – $[C_7H_7]^+$ 161.1317 – $[C_{12}H_{17}]^+$	Tentative identification
Terpenoids	Terpenoid (I)			40.34			93.0693 – $[C_7H_9]^+$ 111.0799 – $[C_7H_{11}O]^+$ 133.1003 – $[C_{10}H_{13}]^+$	Not defined compound
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenoids)	β-Eudesmol	C15H26O	222.1984	40.42		473-15-4	149.1332 – $[C_{11}H_{17}]^+$ 93.0701 – $[C_7H_9]^+$ 79.0544 – $[C_6H_7]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	Selina-3,7(11)-diene	C15H24	204.1868	40.55	93	6813-21-4	161.1318 – $[C_{12}H_{17}]^+$ 107.0849 – $[C_8H_{11}]^+$ 122.1079 – $[C_9H_{14}]^+$	Tentative identification

Continuation Supplementary Table 2

Family	Compound name	Formula	Mass	RT (min)	RI difference*	CAS ID	Fragments	Identification
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	γ-Guaiene	C15H24	204.1866	40.64	100		107.0851 – $[C8H11]^+$ 93.0695 – $[C7H9]^+$ 119.0850 – $[C9H11]^+$	Tentative identification
Terpenoids	Terpenoid (II)			40.76			93.0692 – $[C7H9]^+$ 67.0538 – $[C5H7]^+$ 82.0768 – $[C6H10]^+$	Not defined compound
Terpenoids (sesquiterpenoids, monocyclic sesquiterpenoids)	α-Bisabolol	C15H26O	222.1984	41.02		473-15-4	119.0859 – $[C9H11]^+$ 109.1013 – $[C8H13]^+$ 67.0542 – $[C5H7]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenoids)	γ-Eudesmol	C15H26O	222.1967	41.19	69	1209-71-8	189.1628 – $[C14H21]^+$ 161.1314 – $[C12H17]^+$ 133.1002 – $[C10H13]^+$	Tentative identification
Terpenoids (sesquiterpenoids, acyclic sesquiterpenoids)	*trans-trans*-Farnesol	C15H26O	222.1984	41.44		106-28-5	69.0702 – $[C5H9]^+$ 81.0702 – $[C6H9]^+$ 93.0700 – $[C7H9]^+$	Standard
Terpenoids (sesquiterpenoids, bicyclic sesquiterpenes)	2,6,10,10-Tetramethyl-bicyclo[7.2.0]undeca-2,6-diene	C15H24	204.1864	42.41	100		149.1319 – $[C11H17]^+$ 81.0695 – $[C6H9]^+$ 93.0693 – $[C7H9]^+$	Tentative identification
Terpenoids (diterpenoids)	*trans*-Phytol	C20H40O	296.3079	44.86		150-86-7	71.0498 – $[C4H7O]^+$ 81.0700 – $[C6H9]^+$ 95.0854 – $[C7H11]^+$	Standard
Terpenoids (triterpenoids)	Squalene	C30H50	410.3878	55.18	-94	111-02-4	81.0699 – $[C6H9]^+$ 69.0698 – $[C5H9]^+$ 95.0851 – $[C7H11]^+$	Tentative identification
Terpenoids (triterpenoids)	β-Amyrin	C30H50O	426.3820	61.97	84	559-70-6	203.1793 – $[C15H23]^+$ 218.2027 – $[C16H26]^+$ 189.1635 – $[C14H21]^+$	Tentative identification
Terpenoids (triterpenoids)	α-Amyrin	C30H50O	426.3826	62.73	97	638-95-9	218.2022 – $[C16H26]^+$ 203.1787 – $[C15H23]^+$ 189.1632 – $[C14H21]^+$	Tentative identification

*RI difference = Experimental RI - NIST RI; ** New compound identified in *Cannabis*.

Supplementary Table 3. Identified compounds in polar extracts from *Cannabis* analyzed by LC–QTOF MS/MS and the main parameters that support their identification.

Family	Compound name	Formula	Mass	RT (min)	Precursor ion (*m/z*)	Adduct	Fragments	Identification
Alkaloids	Trigonelline	C7H8NO2	138.0561	4.39	138.0555	$[M]^+$	78.0340 92.0501 94.0654	Tentative identification
Amino acids	Arginine	C6H14N4O2	174.1121	3.44	175.1194	$[M+H]^+$	60.0559 70.0649 116.0713	Tentative identification
Amino acids	Glutamic acid	C5H9NO4	147.0534	4.02	146.0460	$[M-H]^-$	102.0562	Tentative identification
Amino acids	Betaine	C5H12NO2	118.0874	4.20	118.0868	$[M]^+$	58.0655 59.0730	Tentative identification*
Amino acids	Proline	C5H9NO2	115.0633	4.33	116.0706	$[M+H]^+$	68.0499 70.0654	Tentative identification
Amino acids	Phenylalanine	C9H11NO2	165.0799	10.53	166.0872	$[M+H]^+$	77.0381 103.054 120.0805	Tentative identification
Amino acids	Tryptophan	C11H12N2O2	204.0895	11.74	205.0968	$[M+H]^+$	118.0649 144.0804 146.0584	Tentative identification
Carbohydrates (O-glycosyl compounds)	Maltose	C12H22O11	342.1170	4.17	341.1097	$[M-H]^-$	59.0147 89.0246 101.0245	Standard
Carbohydrates (O-glycosyl compounds)	Sucrose	C12H22O11	342.1178	4.34	341.1105	$[M-H]^-$	59.0144 71.0147 89.0246	Standard
Cannabinoids	CBDVA	C20H26O4	330.1831	21.50	329.1758	$[M-H]^-$	311.1658 217.1238 151.0762 283.1710 261.1131 243.1030 107.0499 199.1131	Standard
Cannabinoids	CBDV	C19H26O2	286.1919	21.90	287.1992	$[M+H]^+$	165.0906 287.2008 231.1361 93.0700 107.0857 123.0439	Standard
Cannabinoids	CBDA	C22H30O4	358.2145	23.72	357.2072	$[M-H]^-$	357.2075 339.1969 313.2178	Standard
Cannabinoids	CBG	C21H32O2	316.2407	24.07	317.2480	$[M+H]^+$	193.1223 123.0443 81.0689 95.0856	Standard
Cannabinoids	CBGA	C22H32O4	360.2300	24.30	359.2227	$[M-H]^-$	341.2126 315.2329 297.2220 191.1075	Standard

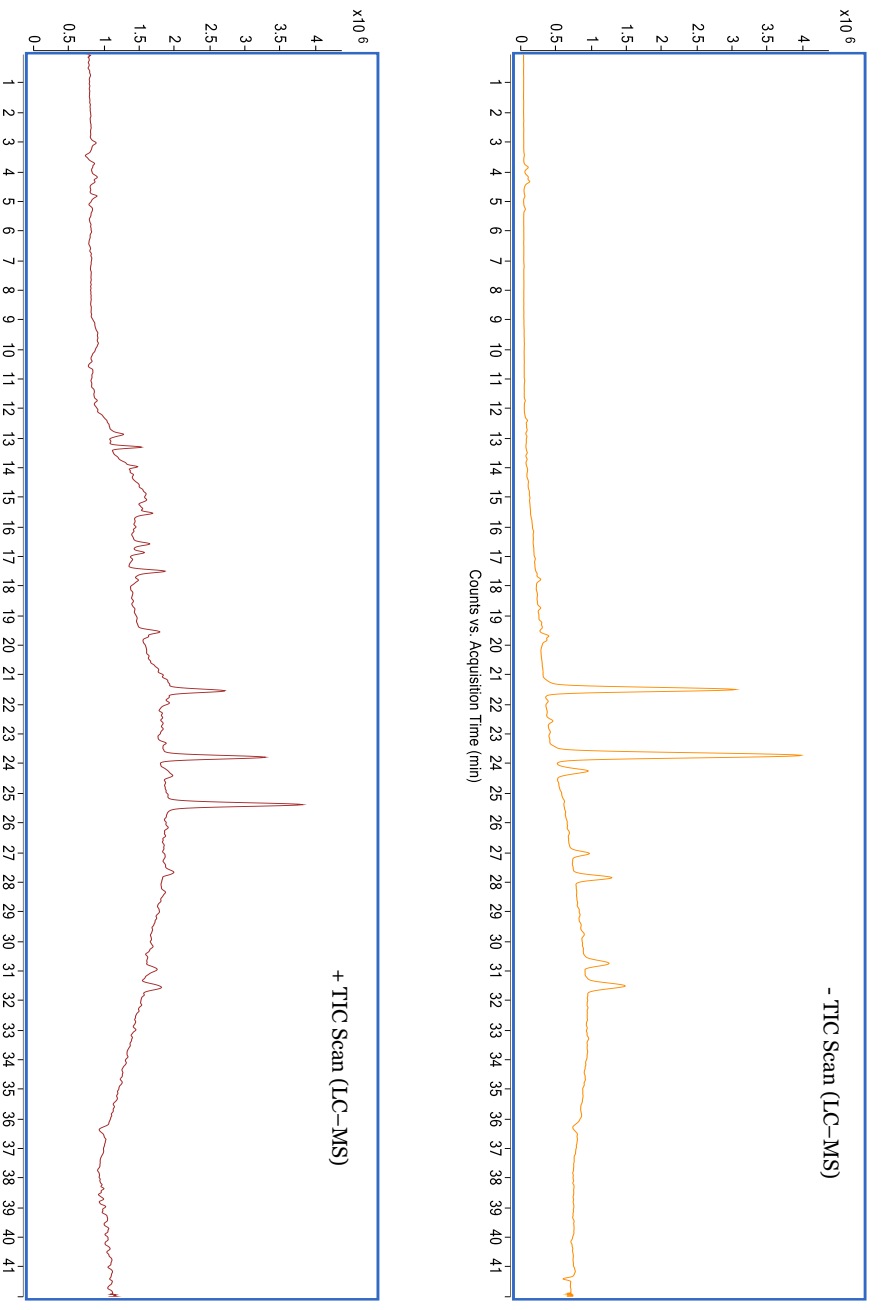
Continuation Supplementary Table 3

Family	Compound name	Formula	Mass	RT (min)	Precursor ion (*m/z*)	Adduct	Fragments	Identification
Cannabinoids	CBD	C21H30O2	314.2258	24.40	315.2331	$[M+H]^+$	193.1228 315.2316 259.1690 135.1169 93.0697 123.0446	Standard
Cannabinoids	THCV	C19H26O2	286.1919	25.10	287.1992	$[M+H]^+$	123.0444 231.1360 165.0907	Standard
Cannabinoids	CBN	C21H26O2	310.1935	26.83	309.1863	$[M-H]^-$	309.1860 279.1399	Standard
Cannabinoids	Δ9-THC	C21H30O2	314.2227	27.50	315.2300	$[M+H]^+$	123.0440 193.1224 259.1691	Standard
Cannabinoids	Δ8-THC	C21H30O2	314.2243	28.30	315.2316	$[M+H]^+$	123.0444 193.1223 259.1689	Standard
Cannabinoids	CBC	C21H30O2	314.2230	29.40	315.2303	$[M+H]^+$	259.1690 81.0689 193.1224	Standard
Cannabinoids	CBL	C21H30O2	314.2246	29.50	313.2173	$[M-H]^-$	191.1075 204.1146 179.1087	Standard
Cannabinoids	THCA	C22H30O4	358.2144	30.80	357.2071	$[M-H]^-$	313.2177 245.1543 191.1078 339.1941 179.1085	Standard
Flavonoids (flavanols)	Procyanidin B	C30H26O12	578.1430	12.27	577.1357	$[M-H]^-$	125.0251 289.0741 407.0772	Not exactly defined compound*
Flavonoids (flavanols)	Procyanidin C	C45H38O18	866.2055	12.29	865.1983	$[M-H]^-$	407.0781 575.1223 713.1497	Not exactly defined compound*
Flavonoids (flavonols)	Kaempferol-rutinoside (isomer)	C27H30O15	594.1596	12.42	593.1515	$[M-H]^-$	327.0527 357.0601	Not exactly defined compound
Flavonoids (flavanols)	Procyanidin B2	C30H26O12	578.1432	12.43	577.1357	$[M-H]^-$	273.0408 289.0710 425.0869	Standard*
Flavonoids (flavanols)	Procyanidin C	C45H38O18	866.2049	12.49	865.1977	$[M-H]^-$	425.0844 575.1166 695.1400	Not exactly defined compound*
Flavonoids (flavones)	Vitexin-2-rhamnoside	C27H30O14	578.1652	12.49	577.1579	$[M-H]^-$	293.0440 413.0898	Standard
Flavonoids (flavones)	Luteolin-6-C-glucoside (Homoorientin)	C21H20O11	448.1006	12.55	447.0941	$[M-H]^-$	327.0514 357.0643	Tentative identification
Flavonoids (flavones)	Vitexin	C21H20O10	432.1029	12.67	433.1102	$[M+H]^+$	397.0855 415.0968 313.0683	Standard
Flavonoids (flavonols)	Kaempferol-7-O-glucoside (2 peaks)	C21H20O11	448.1019	12.72 12.92	447.0947	$[M-H]^-$	284.0327 285.0429 298.0525	Standard
Flavonoids (flavonols)	Quercetin 3-galactoside	C21H20O12	464.0957	12.75	463.0884	$[M-H]^-$	300.0278 301.0344	Standard
Flavonoids (flavones)	Apigenin 7-O-glucoside	C21H20O10	432.1065	12.96	431.0992	$[M-H]^-$	268.0390 269.0468	Tentative identification

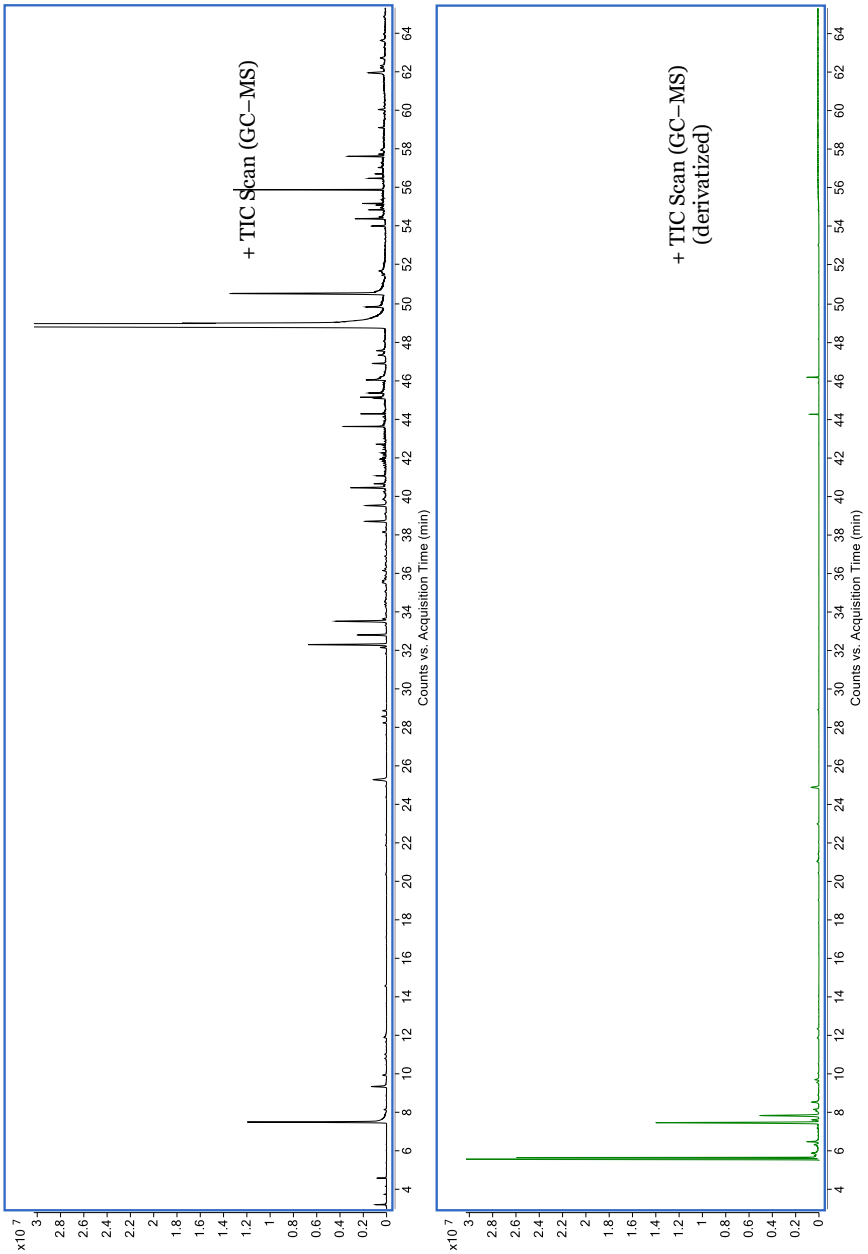
Continuation Supplementary Table 3

Family	Compound name	Formula	Mass	RT (min)	Precursor ion (*m/z*)	Adduct	Fragments	Identification
Flavonoids (flavones)	Baicalin	C21H18O11	446.0855	13.04	445.0782	$[M-H]^-$	85.0297 113.0239 269.0459	Tentative identification
Flavonoids (flavones)	Luteolin	C15H10O6	286.0468	14.38	287.0541	$[M+H]^+$	67.0180 153.0171 161.0222	Standard
Flavonoids (flavonols)	Quercetin	C15H10O7	302.0434	14.44	301.0361	$[M-H]^-$	121.0296 151.0033 178.9984	Standard
Flavonoids (flavones)	Apigenin	C15H10O5	270.0531	15.13	269.0458	$[M-H]^-$	107.0133 117.0360 149.0229	Standard
Flavonoids (flavones)	Diosmetin	C16H12O6	300.0634	15.35	299.0561	$[M-H]^-$	256.0384 284.0322 299.0530	Standard
Lipids (glycerphospholipid)	Glycerophospho-choline	C8H21NO6P	258.1102	4.05	258.1097	$[M]^+$	104.1078 124.9995 184.0730	Tentative identification*
Lipids (long-chain fatty acids)	Ricinoleic acid	C18H34O3	298.2512	22.78	297.2439	$[M-H]^-$	183.1395	Tentative identification*
Lipids (long-chain fatty acids)	Pinolenic acid (C18:3)	C18H30O2	278.2249	27.25	277.2176	$[M-H]^-$	59.0144	Tentative identification*
Lipids (steroid ester)	17β-Hydroxyandrost-4-ene-3,11-dione propionate	C22H30O4	358.2149	31.43	357.2076	$[M-H]^-$	313.2173 339.1963	Tentative identification*
Organic acid (urea)	N,N'-Dicyclohexylurea	C13H24N2O	224.1892	17.53	225.1965	$[M+H]^+$	55.0543 83.0848 100.1113	Tentative identification*
Organoheterocyclic compound (indole)	4-Formyl indole	C9H7NO	145.0526	11.75	146.0598	$[M+H]^+$	91.0543 118.0636 119.0677	Tentative identification*
Organonitrogen compound (quaternary ammonium)	Choline	C5H14NO	104.1080	3.75	104.1074	$[M]^+$	45.0344 58.0650 60.0809	Tentative identification
Terpenoid (phenolic diterpene)	Carnosol	C20H26O4	330.1835	18.32	329.1762	$[M-H]^-$	285.1869	Tentative identification*

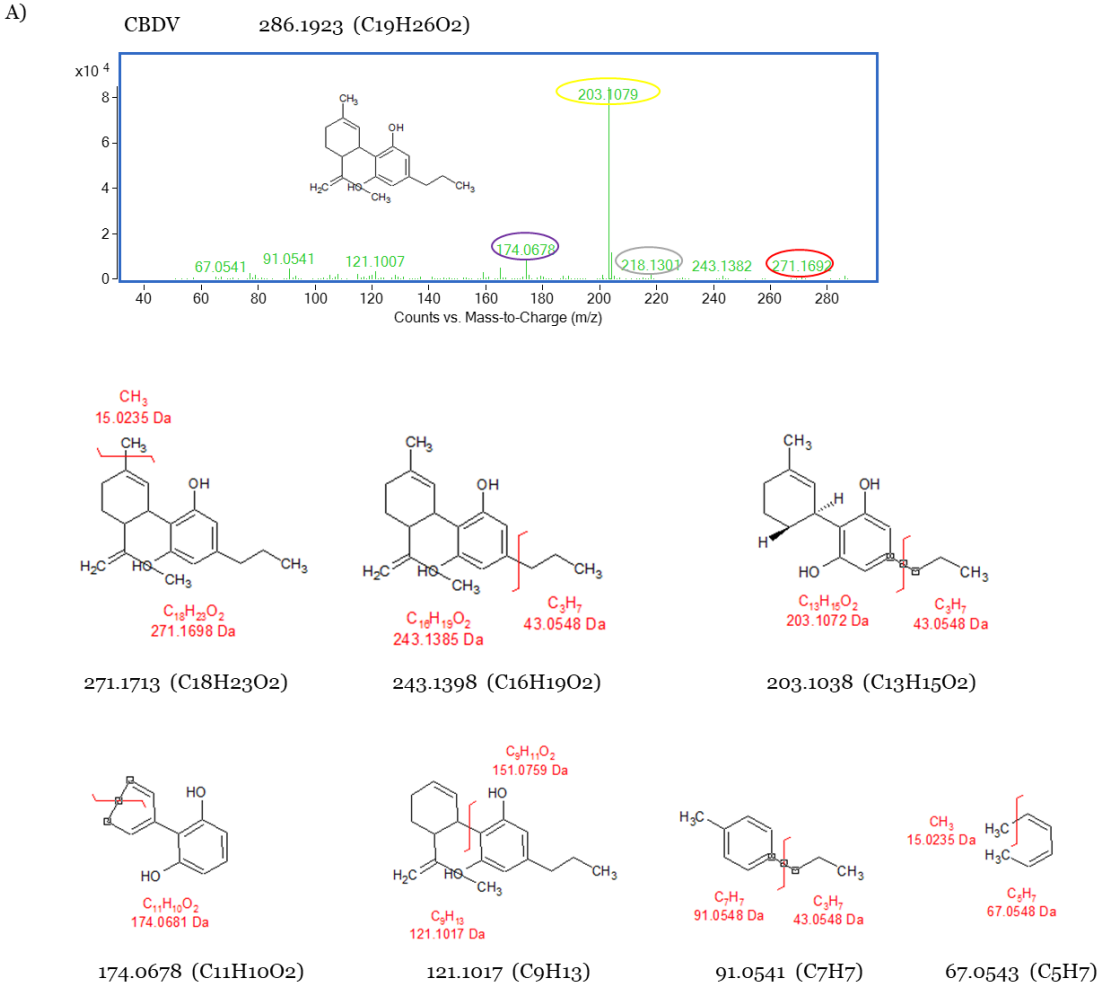
* New compound identified in *Cannabis*.



Supplementary Fig. 1. MS total ion chromatograms from the polar extract of *Cannabis* in positive and negative ionization modes.



Supplementary Fig. 2. MS total ion chromatograms from the no polar extracts of *Cannabis* either derivatized or underivatized before analysis by GC–TOF/MS.

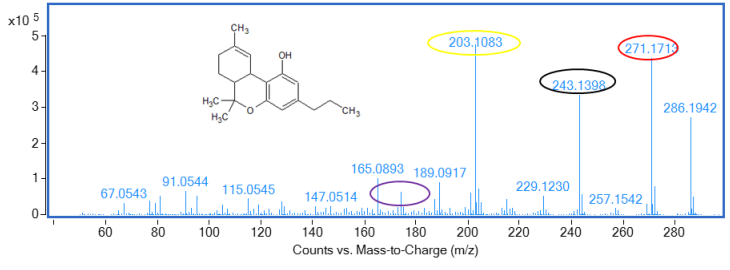


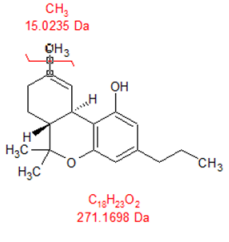
Supplementary Fig. 3. MS spectra of the most common cannabinoids after direct injection of their standards in GC–MS.

Continuation Supplementary Fig. 3

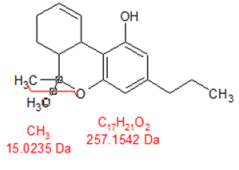
B)

Δ9-THCV 286.1942 ($C_{19}H_{26}O_2$)

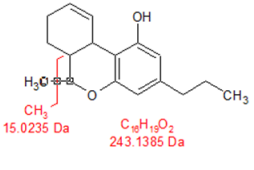




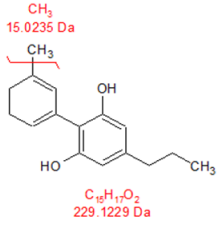
271.1713 ($C_{18}H_{23}O_2$)



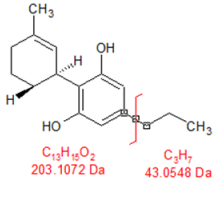
257.1542 ($C_{17}H_{21}O_2$)



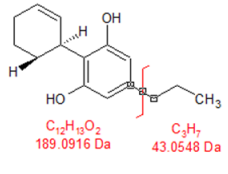
243.1398 ($C_{16}H_{19}O_2$)



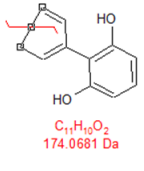
229.1230 ($C_{15}H_{17}O_2$)



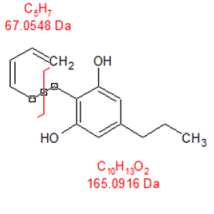
203.1038 ($C_{13}H_{15}O_2$)



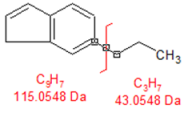
189.0917 ($C_{12}H_{13}O_2$)



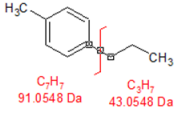
174.0678 ($C_{11}H_{10}O_2$)



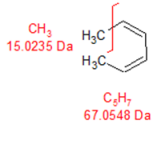
165.0893 ($C_{10}H_{13}O_2$)



115.0545 (C_9H_7)

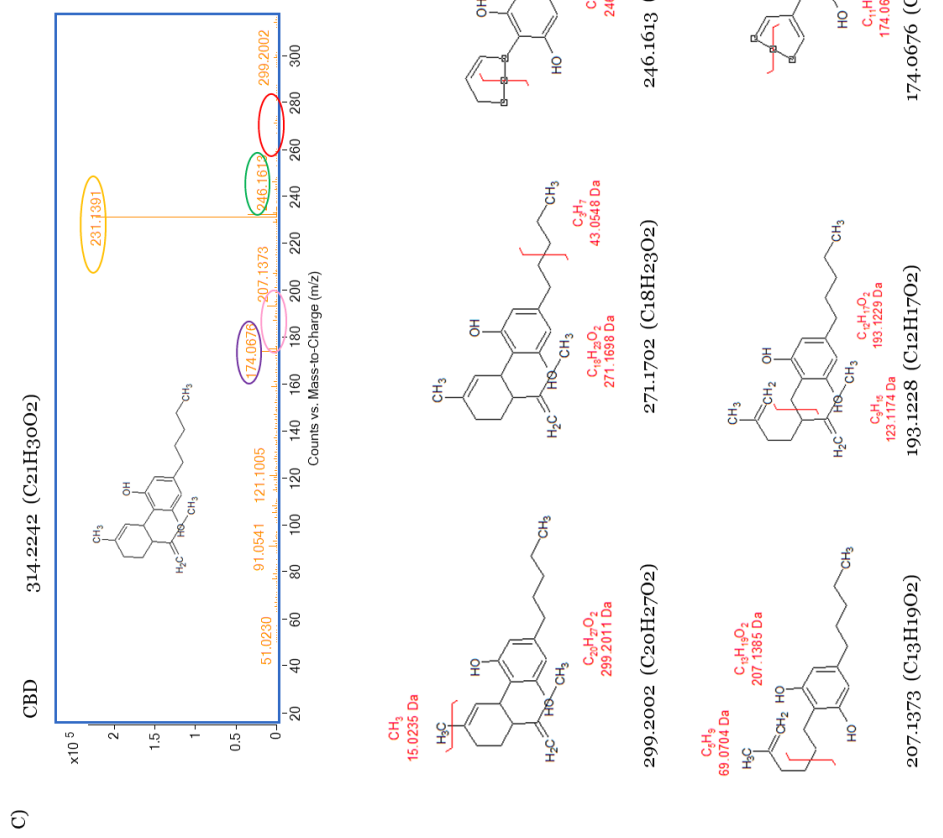


91.0541 (C_7H_7)



67.0543 (C_5H_7)

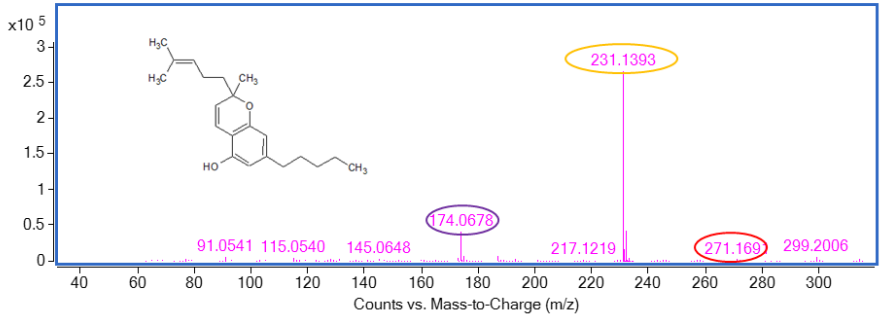
Continuation Supplementary Fig. 3

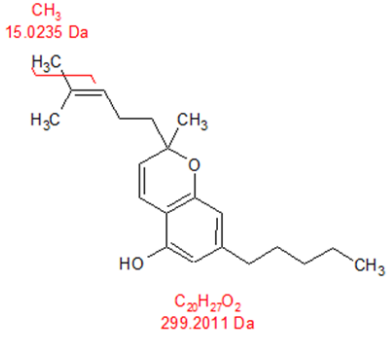


Continuation Supplementary Fig. 3

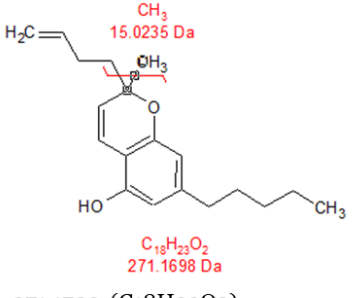
D)

CBC 314.2221 (C21H30O2)

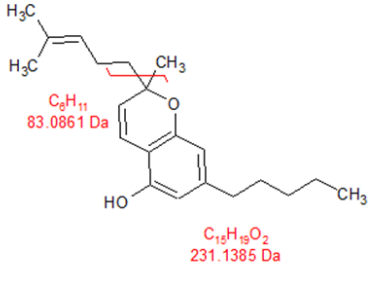




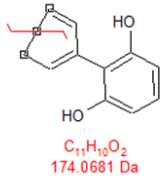
299.2006 (C20H27O2)



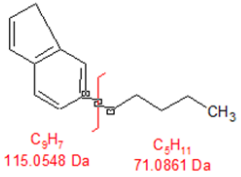
271.1702 (C18H23O2)



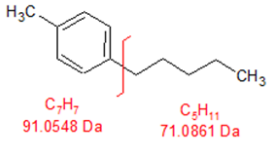
231.1393 (C15H19O2)



174.0678 (C11H10O2)

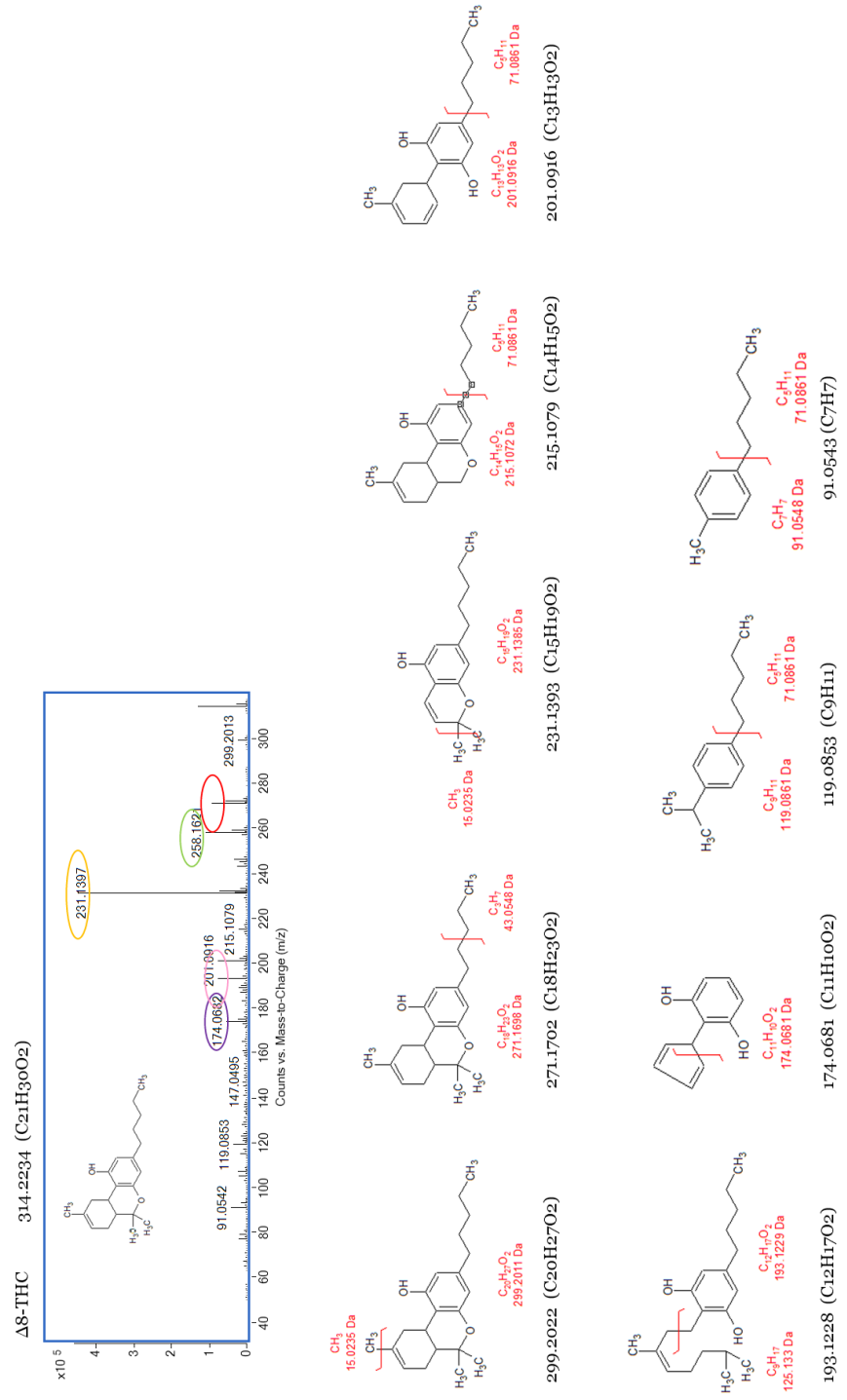


115.0540 (C9H7)



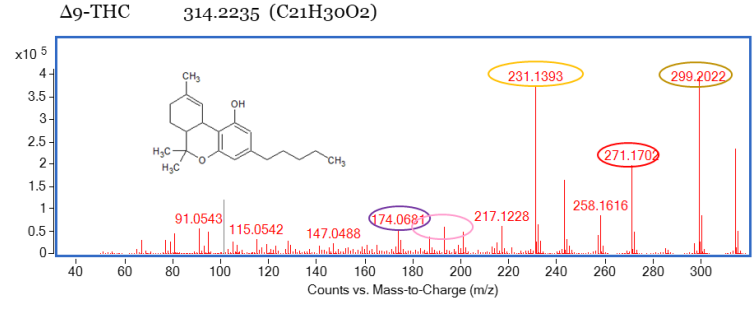
91.0541 (C7H7)

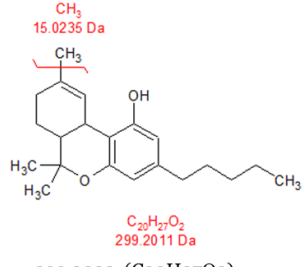
Continuation Supplementary Fig. 3



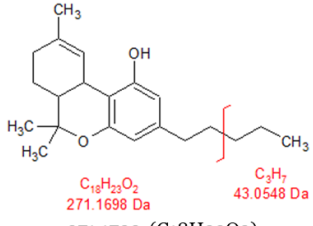
Continuation Supplementary Fig. 3

F)

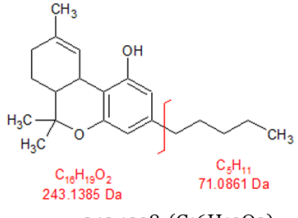




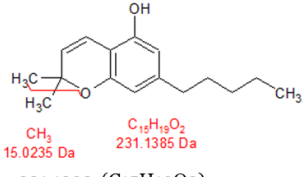
299.2022 (C20H27O2)



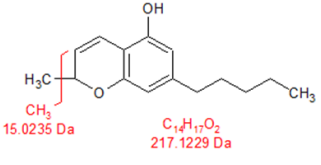
271.1702 (C18H23O2)



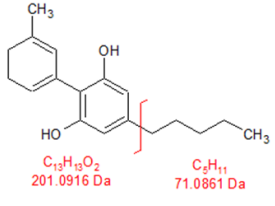
243.1398 (C16H19O2)



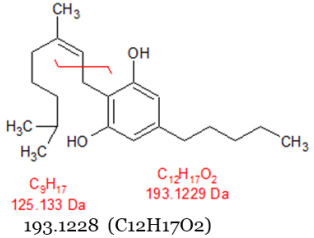
231.1393 (C15H19O2)



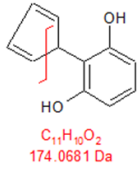
217.1229 (C14H17O2)



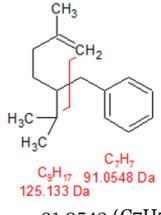
201.0916 (C13H13O2)



193.1228 (C12H17O2)



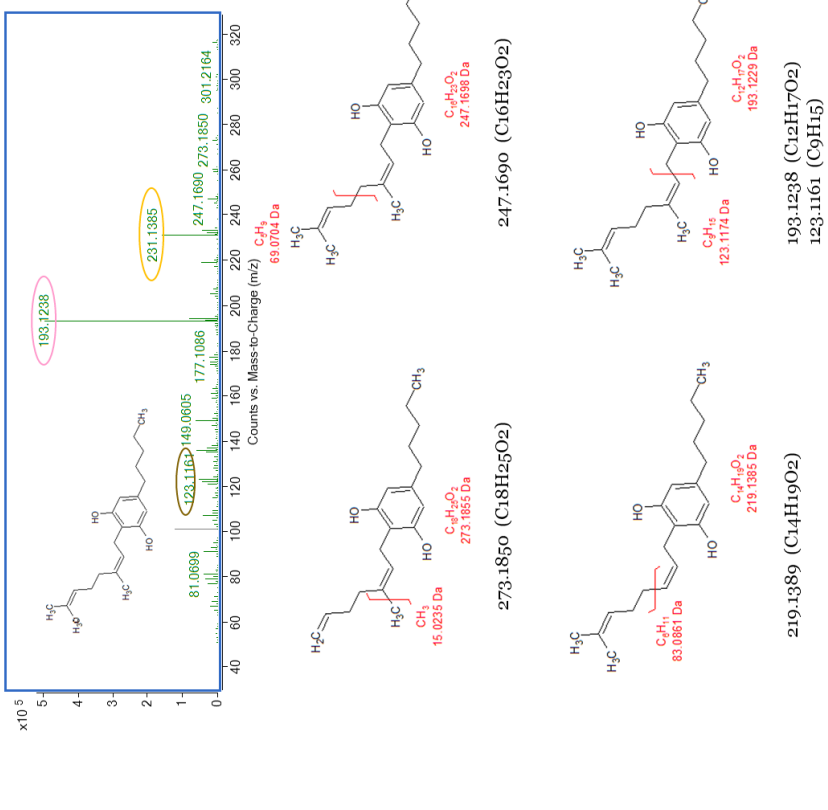
174.0681 (C11H10O2)



91.0543 (C7H7)

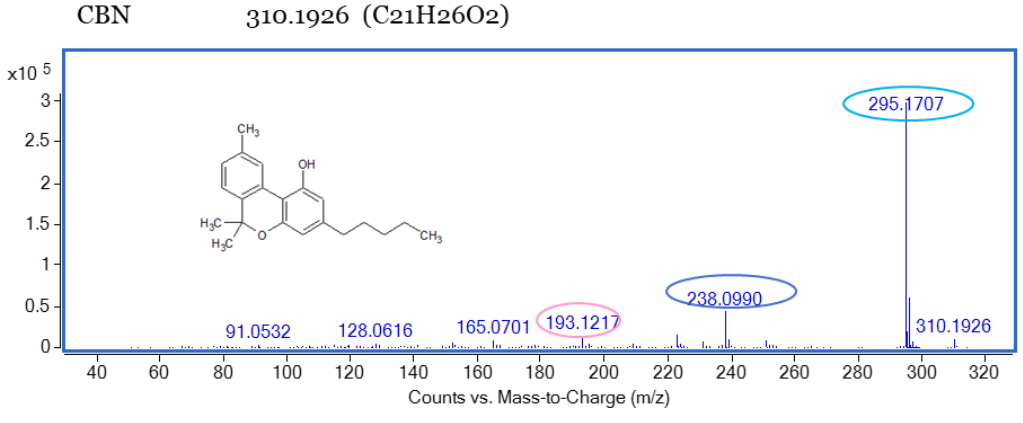
Continuation Supplementary Fig. 3

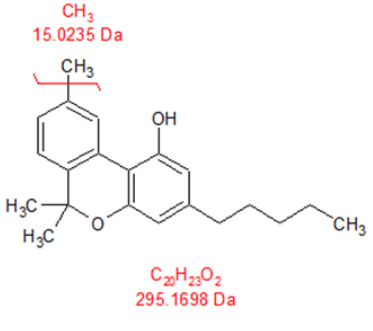
G)



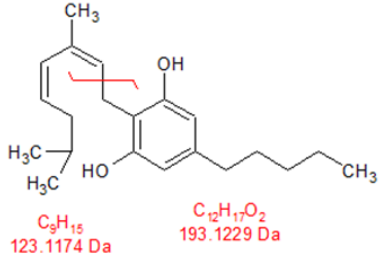
Continuation Supplementary Fig. 3

H)

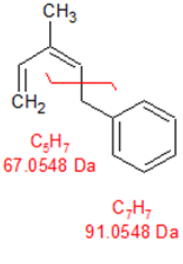




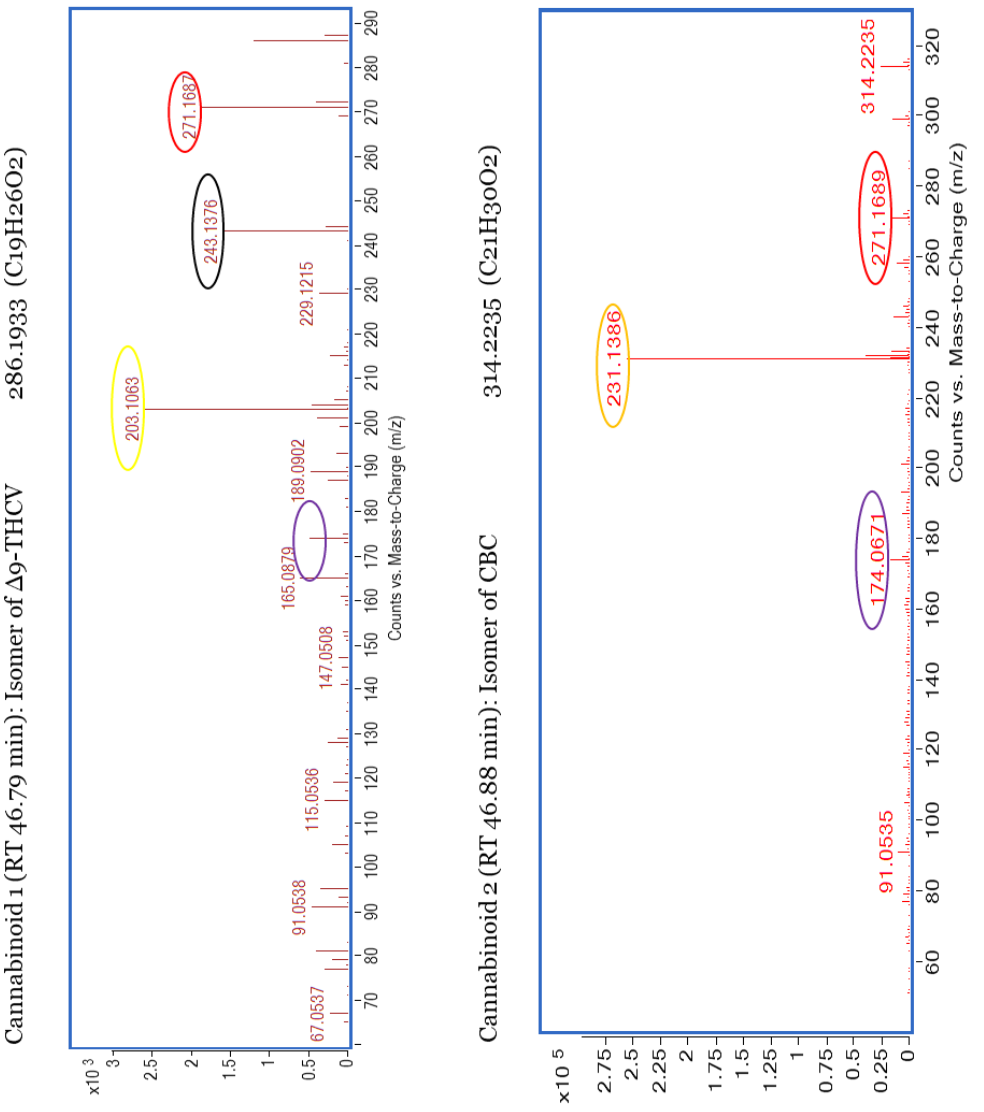
295.1707 (C20H23O2)



193.1229 (C12H17O2)



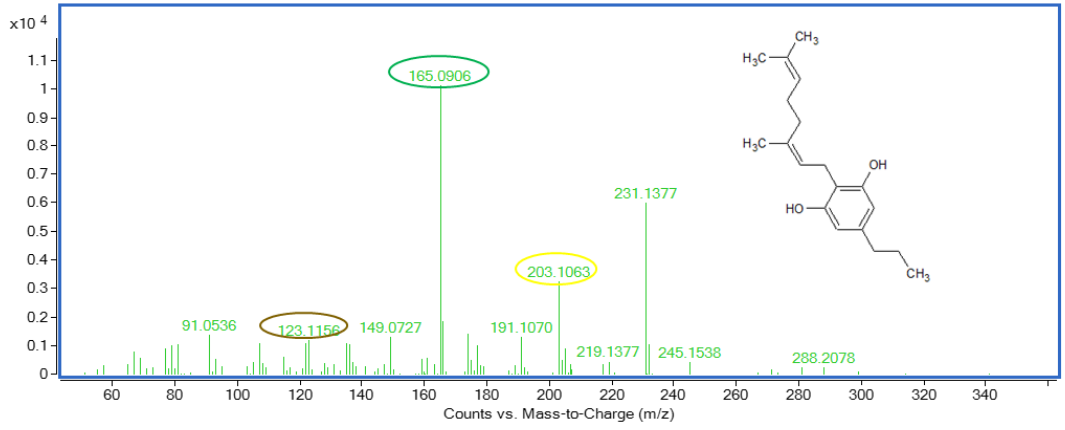
91.0532 (C7H7)



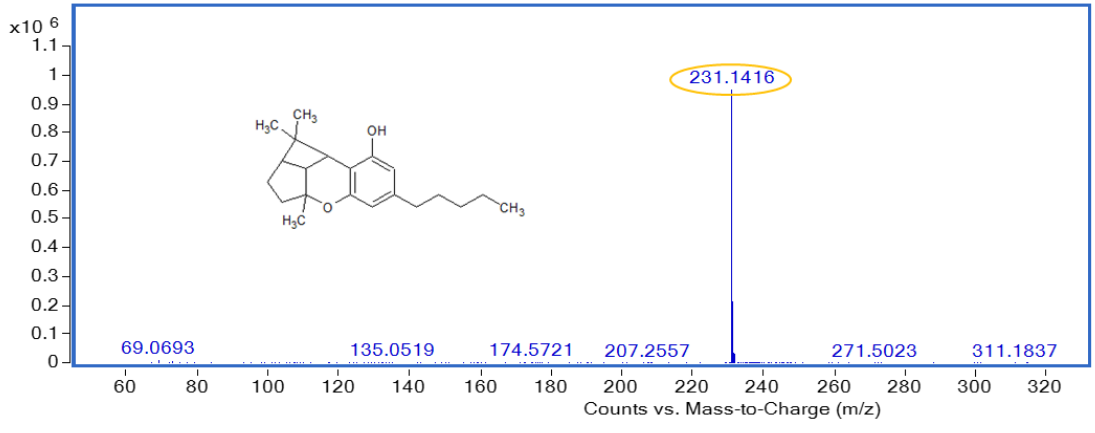
Supplementary Fig. 4. MS spectra of the cannabinoids tentatively identified in no polar *Cannabis* extracts by direct injection in GC–TOF/MS.

Continuation Supplementary Fig. 4

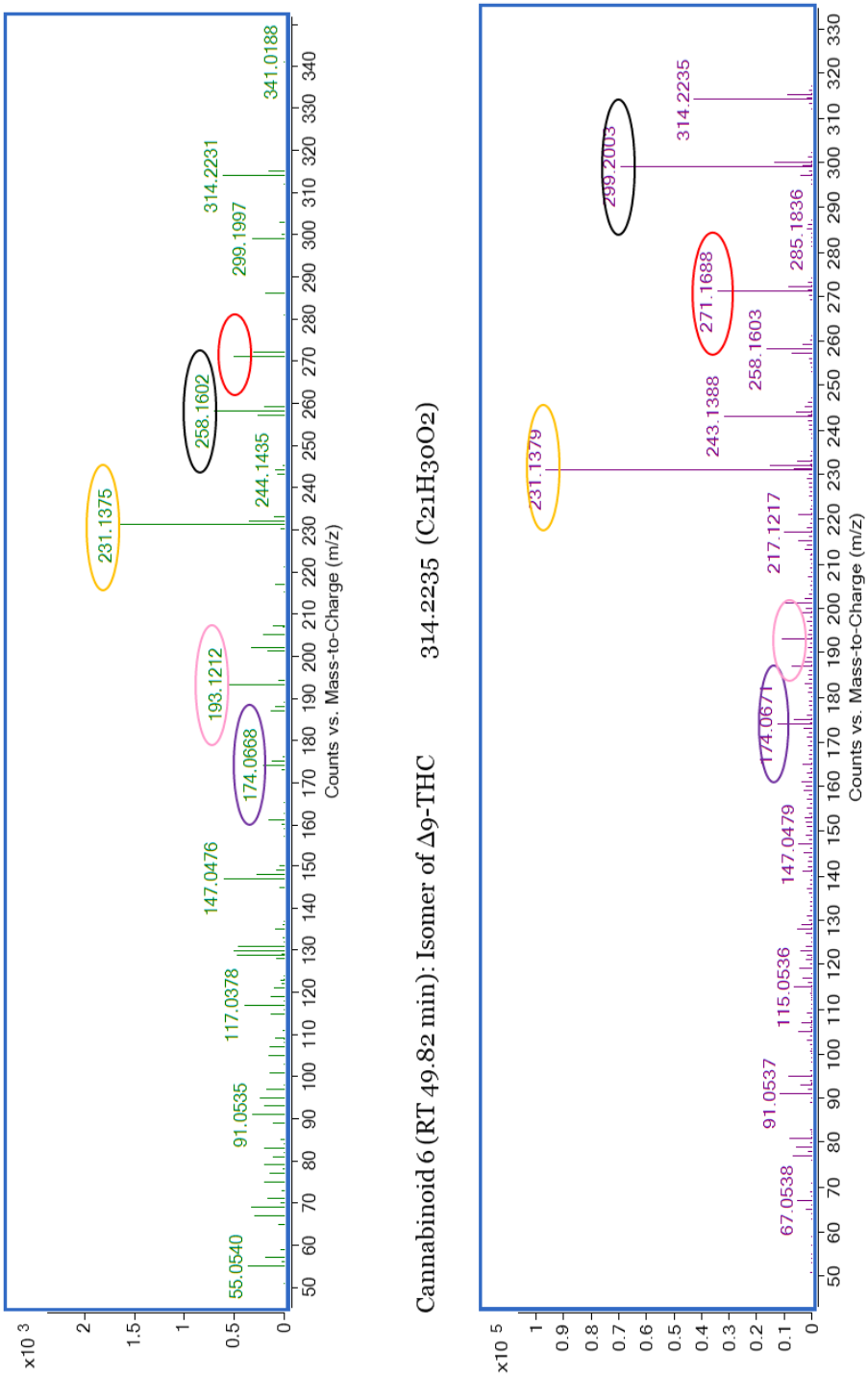
Cannabinoid 3 (RT 48.02 min): CBGV 288.2078 (C19H28O2)



Cannabinoid 4 (RT 48.97 min): CBL 314.2246 (C21H30O2)

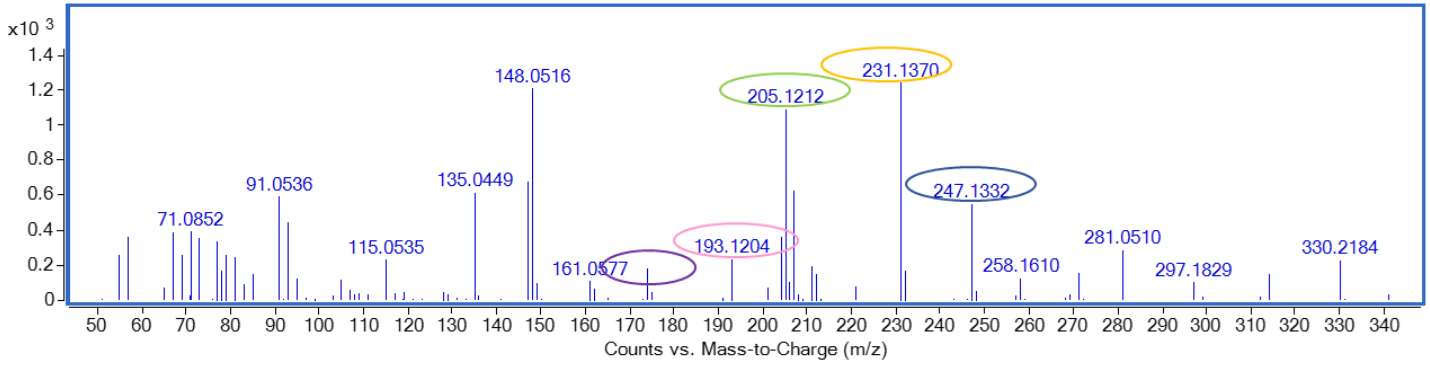


Continuation Supplementary Fig. 4

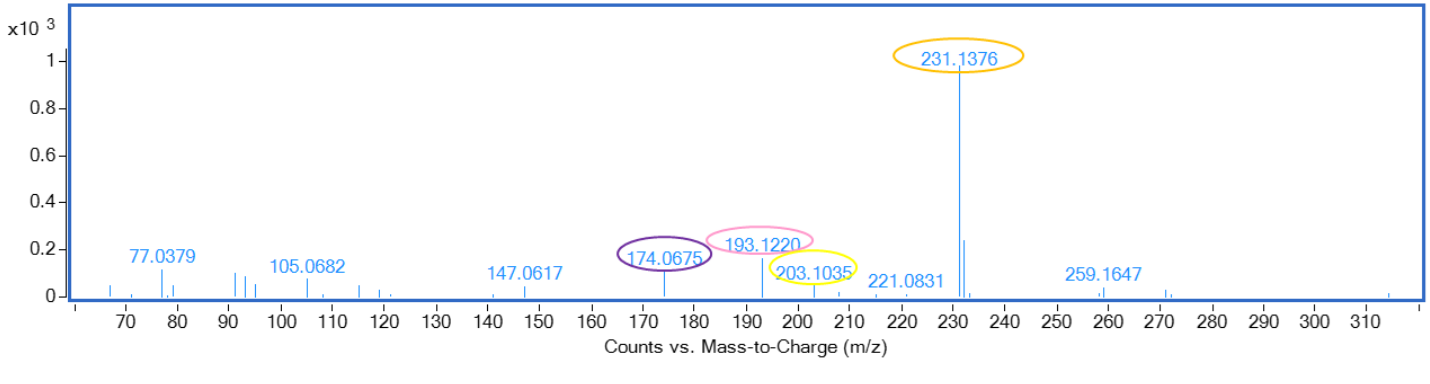


Continuation Supplementary Fig. 4

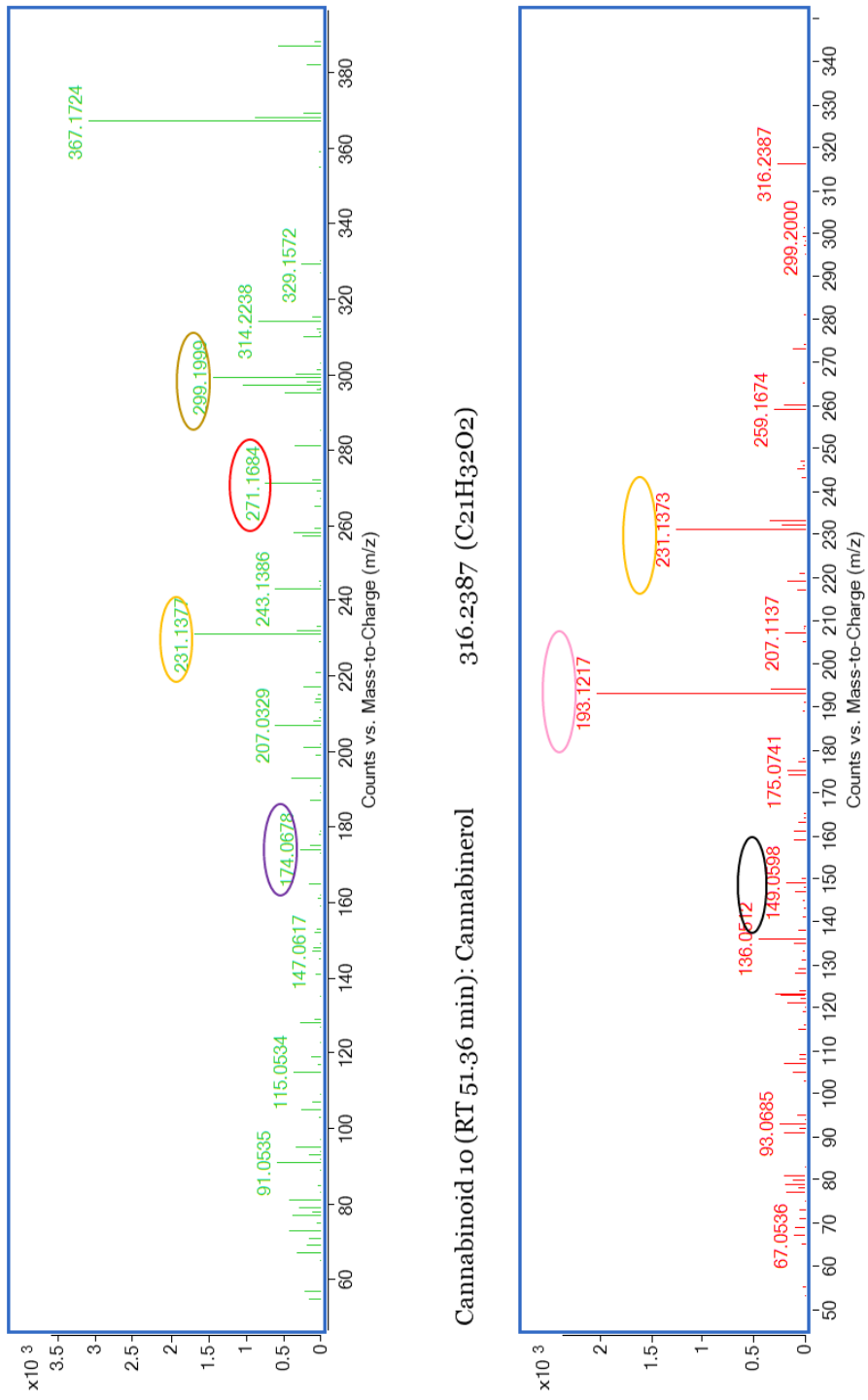
Cannabinoid 7 (RT 50.03 min): CBE 330.2184 (C21H30O3)



Cannabinoid 8 (RT 50.15 min): Isomer of CBC 314.2243 (C21H30O2)

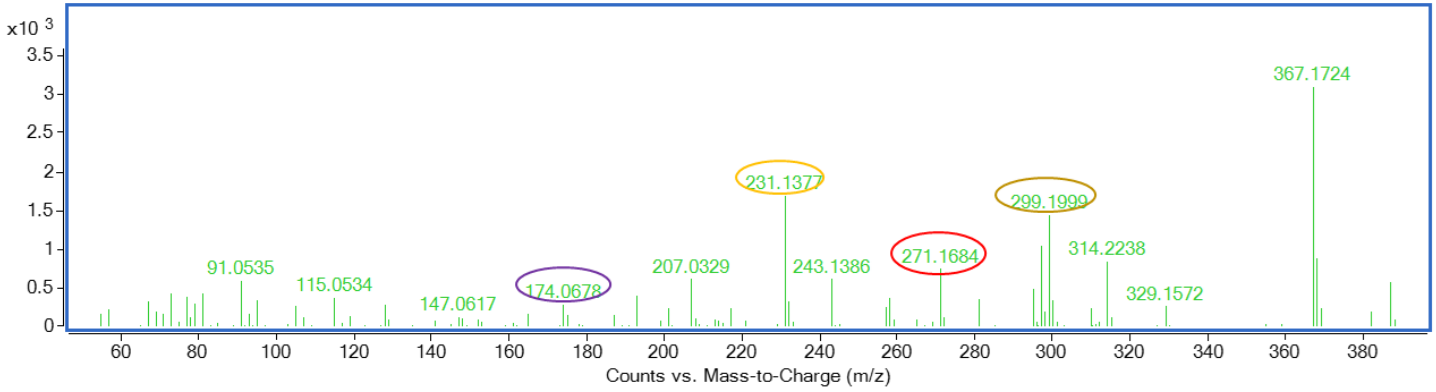


Continuation Supplementary Fig. 4

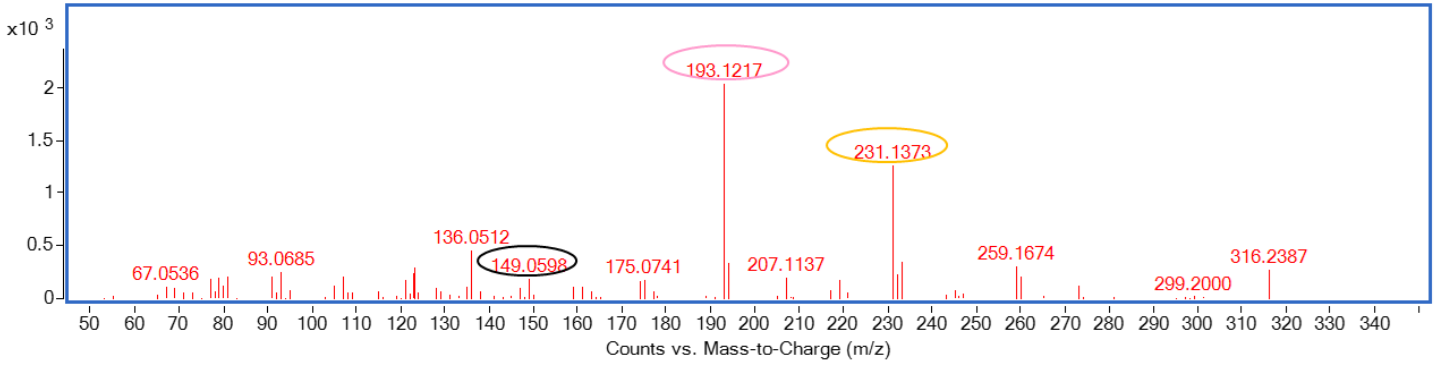


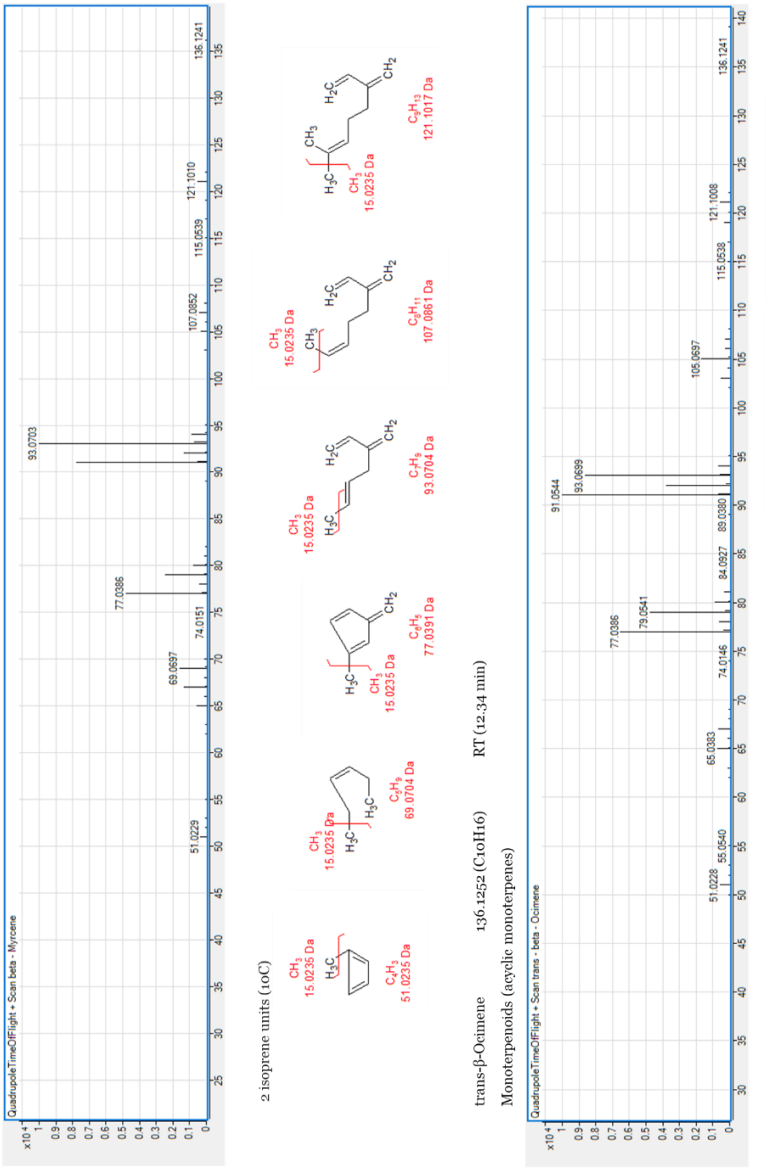
Continuation Supplementary Fig. 4

Cannabinoid 9 (RT 51.04 min): Isomer of Δ9-THC 314.2238 (C21H30O2)



Cannabinoid 10 (RT 51.36 min): Cannabinerol 316.2387 (C21H32O2)



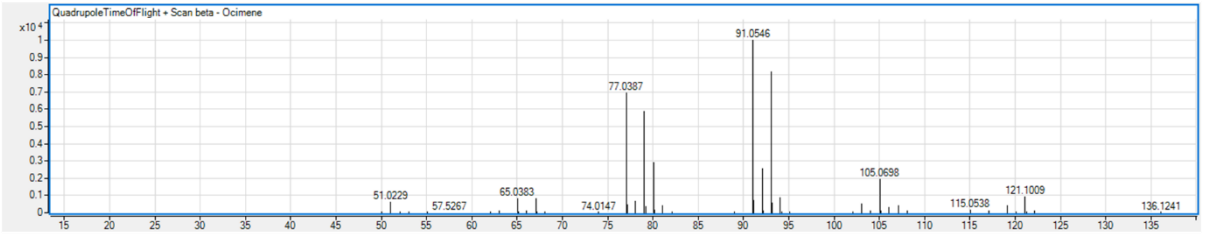


Supplementary Fig. 5. MS spectra of the terpenoids identified in no polar *Cannabis* extracts by direct injection of their standards in GC–TOF/MS.

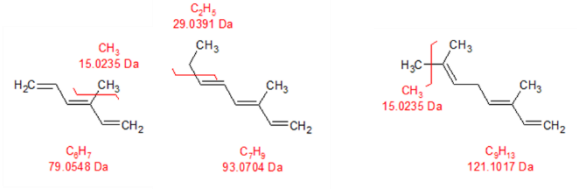
Continuation Supplementary Fig. 5

β -Ocimene 136.1252 (C10H16) RT (12.91 min)

Monoterpenoids (acyclic monoterpenes)

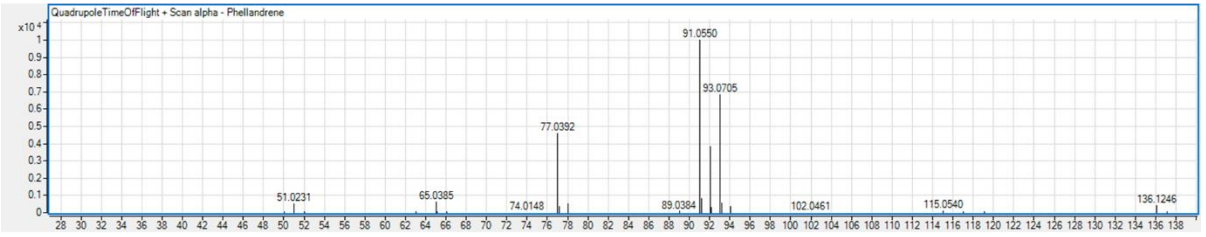


2 isoprene units (10C)

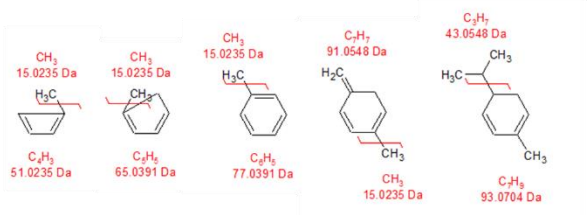


α-Phellandrene 136.1252 (C10H16) RT (10.73 min)

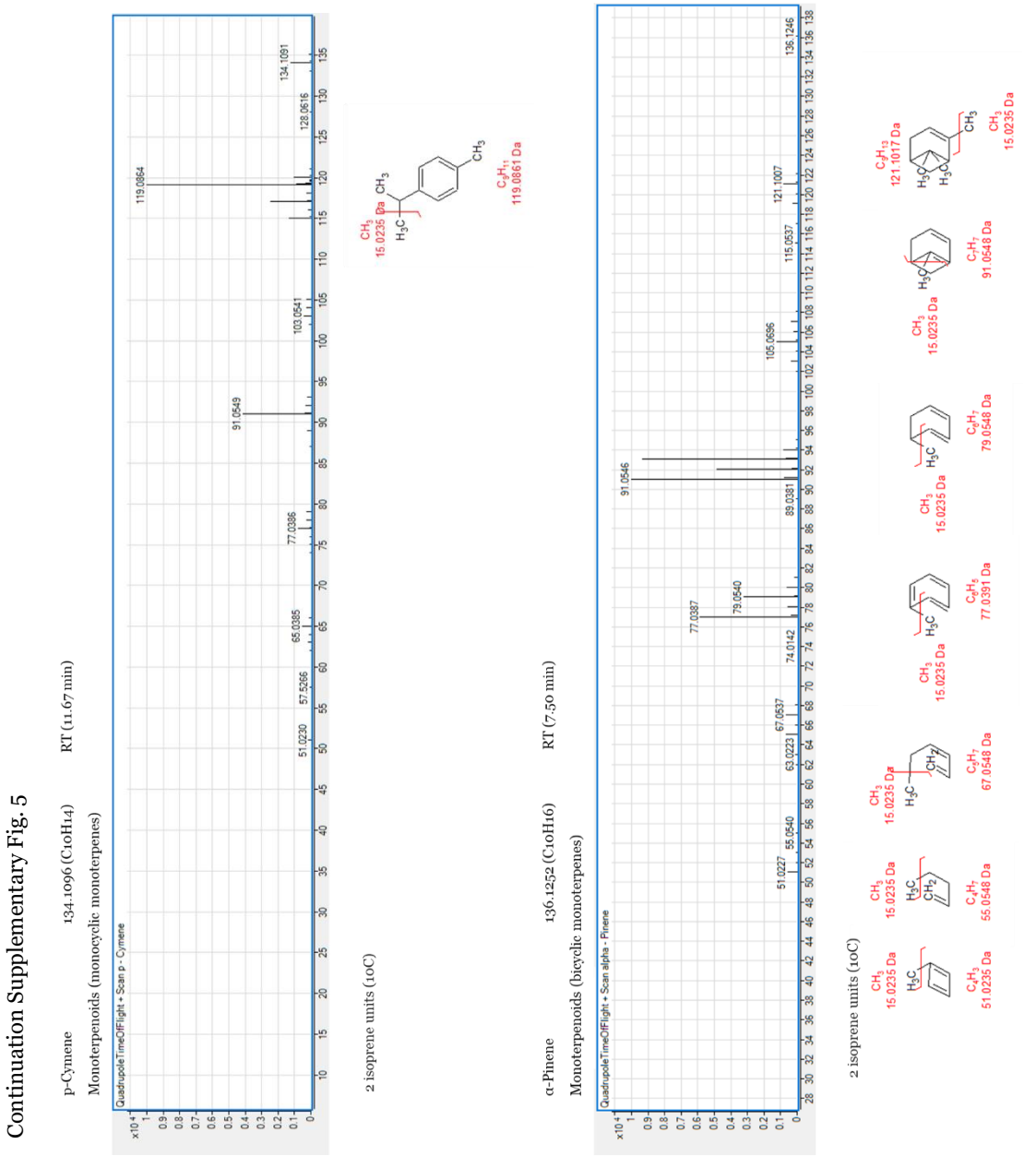
Monoterpenoids (monocyclic monoterpenes)



2 isoprene units (10C)



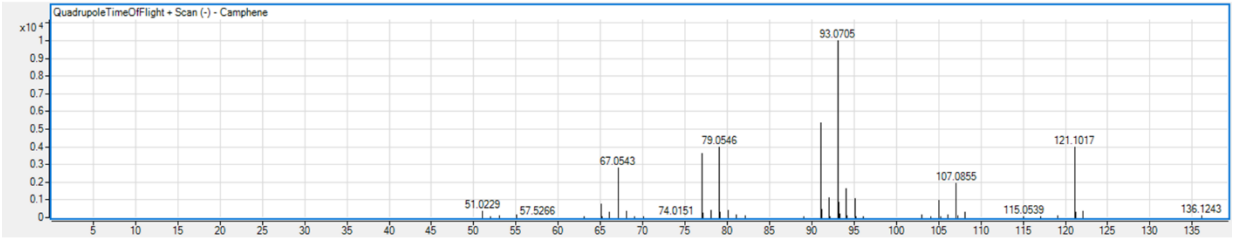
Continuation Supplementary Fig. 5



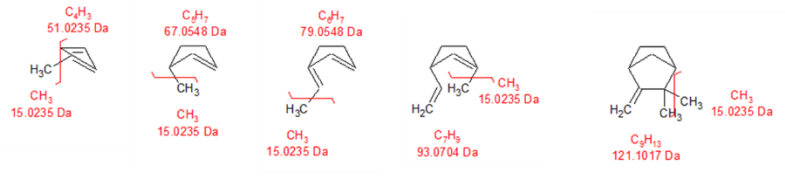
Continuation Supplementary Fig. 5

(-)-Camphene 136.1252 (C10H16) RT (8.16 min)

Monoterpenoids (bicyclic monoterpenes)

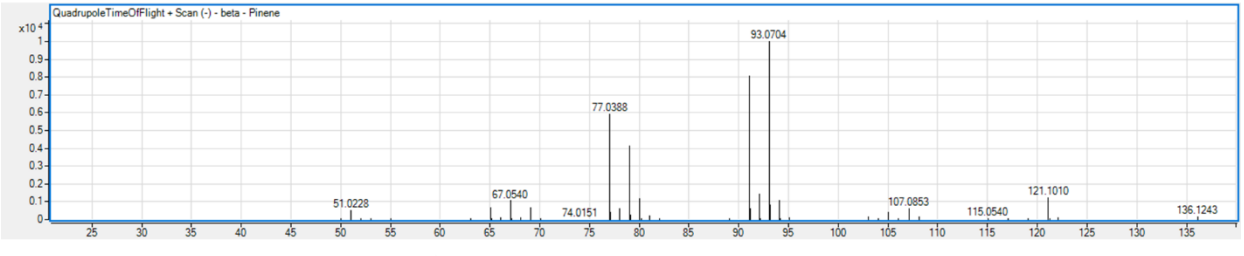


2 isoprene units (10C)

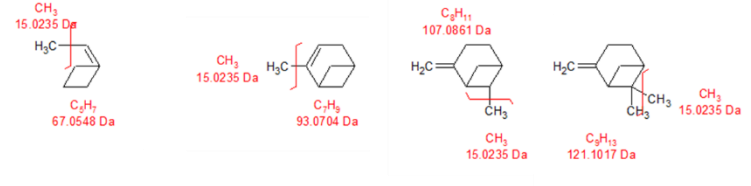


(-)-β-Pinene 136.1252 (C10H16) RT (9.35 min)

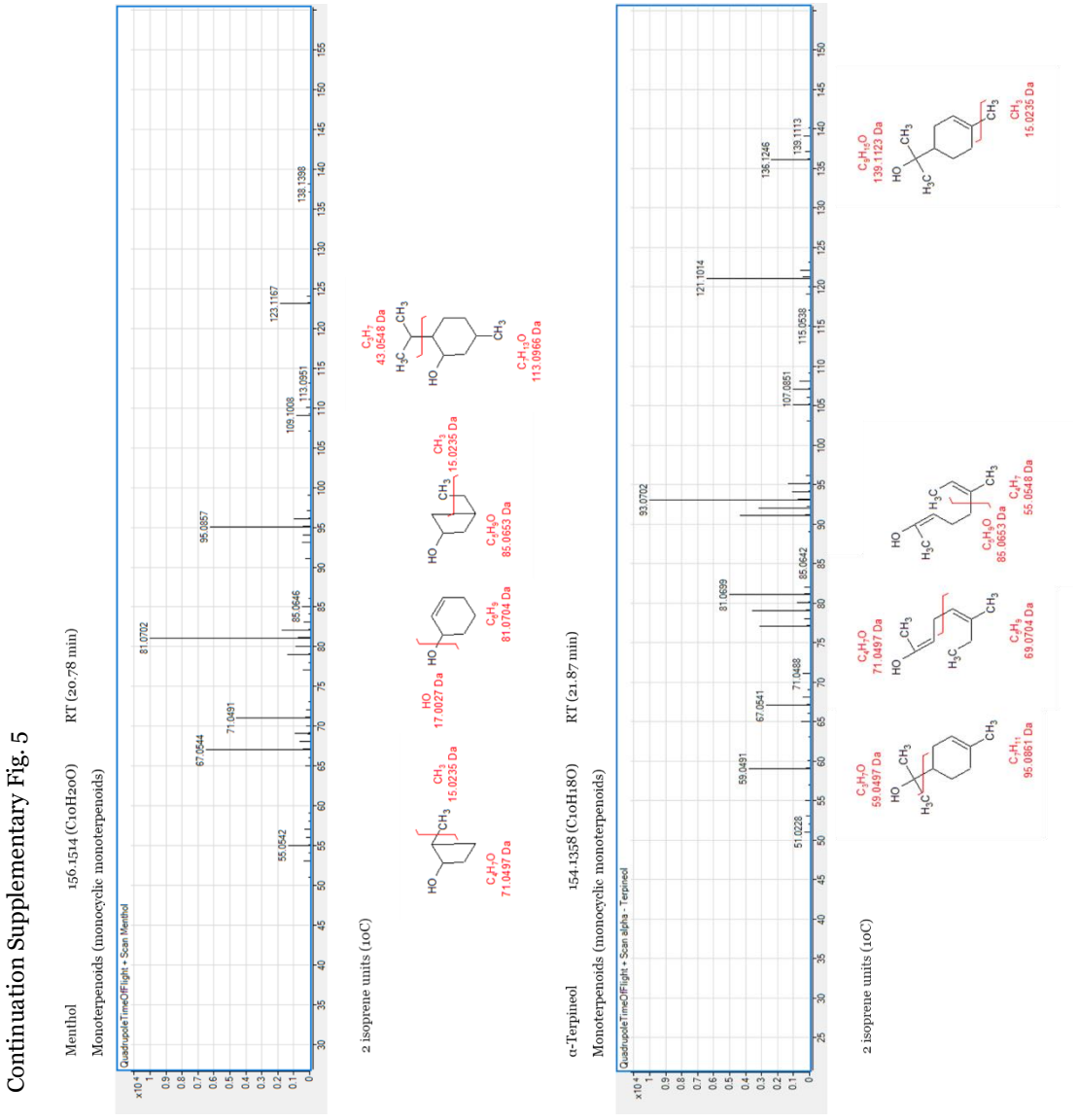
Monoterpenoids (bicyclic monoterpenes)



2 isoprene units (10C)



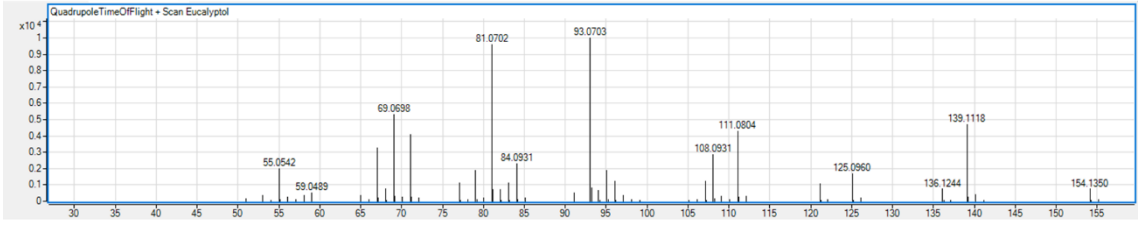
Continuation Supplementary Fig. 5



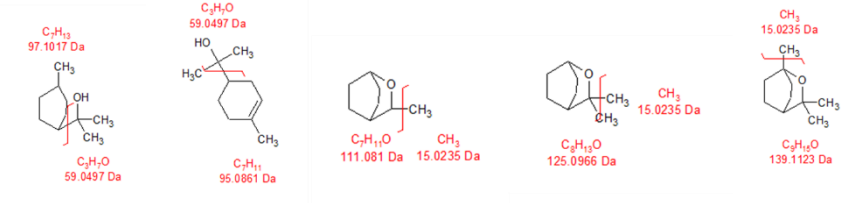
Continuation Supplementary Fig. 5

Eucalyptol 154.1358 (C10H18O) RT (12.04 min)

Monoterpenoids (bicyclic monoterpenoids)

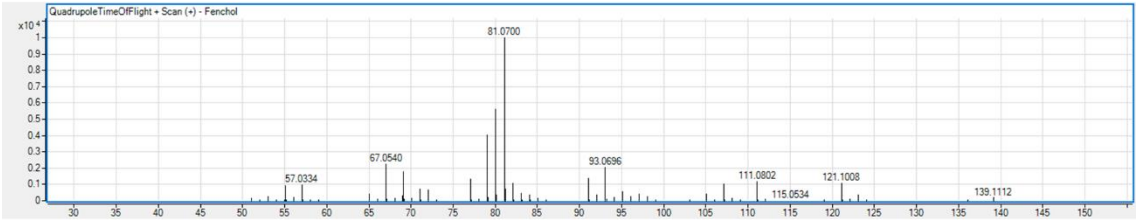


2 isoprene units (10C)

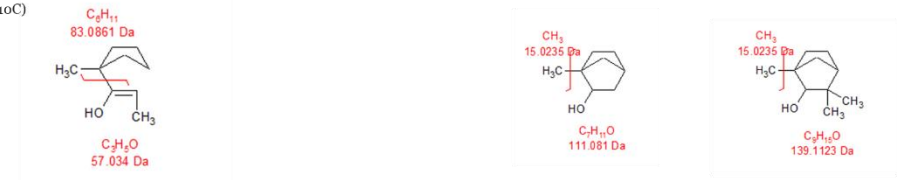


(+)-Fenchol 154.1358 (C10H18O) RT (17.05 min)

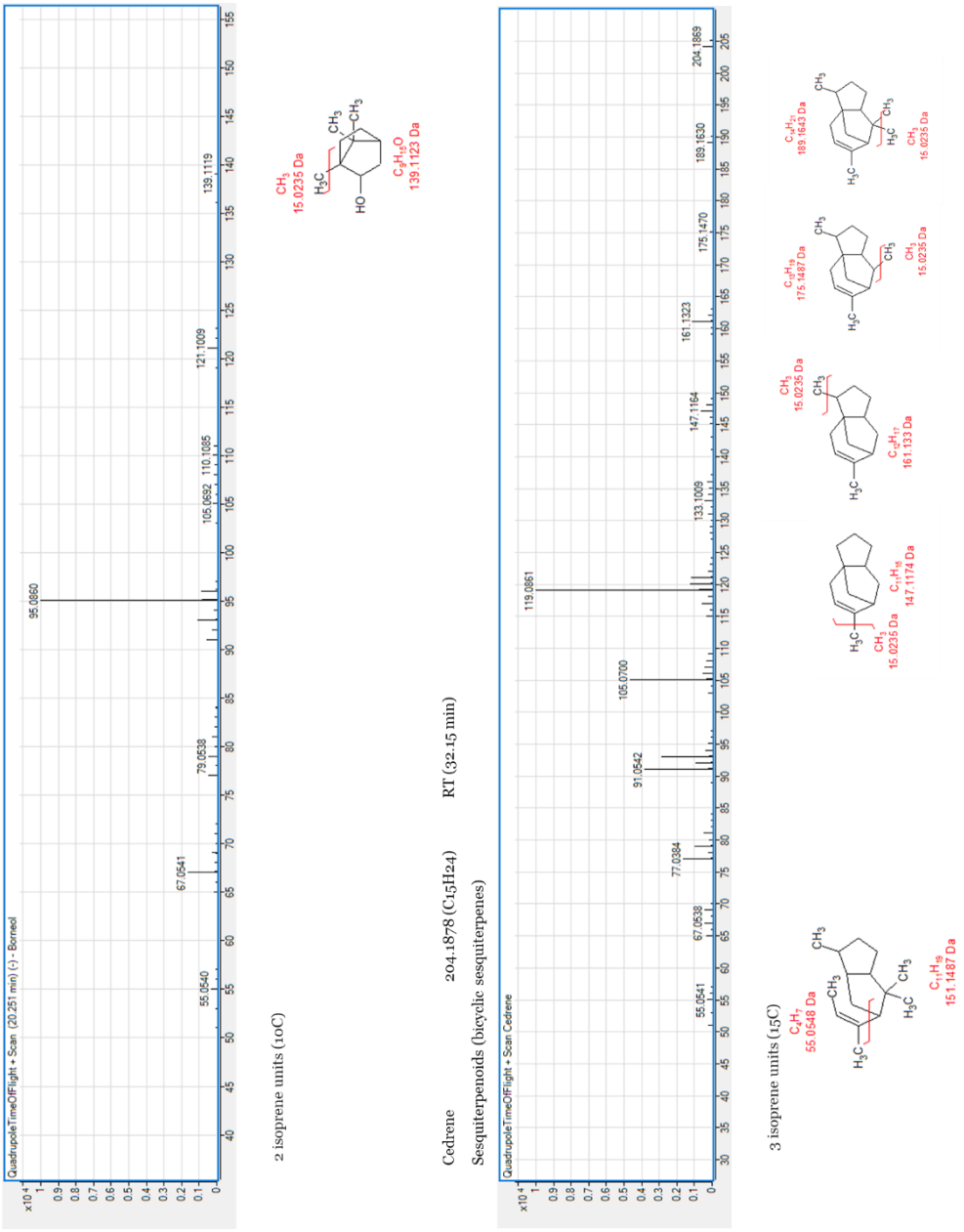
Monoterpenoids (bicyclic monoterpenoids)



2 isoprene units (10C)



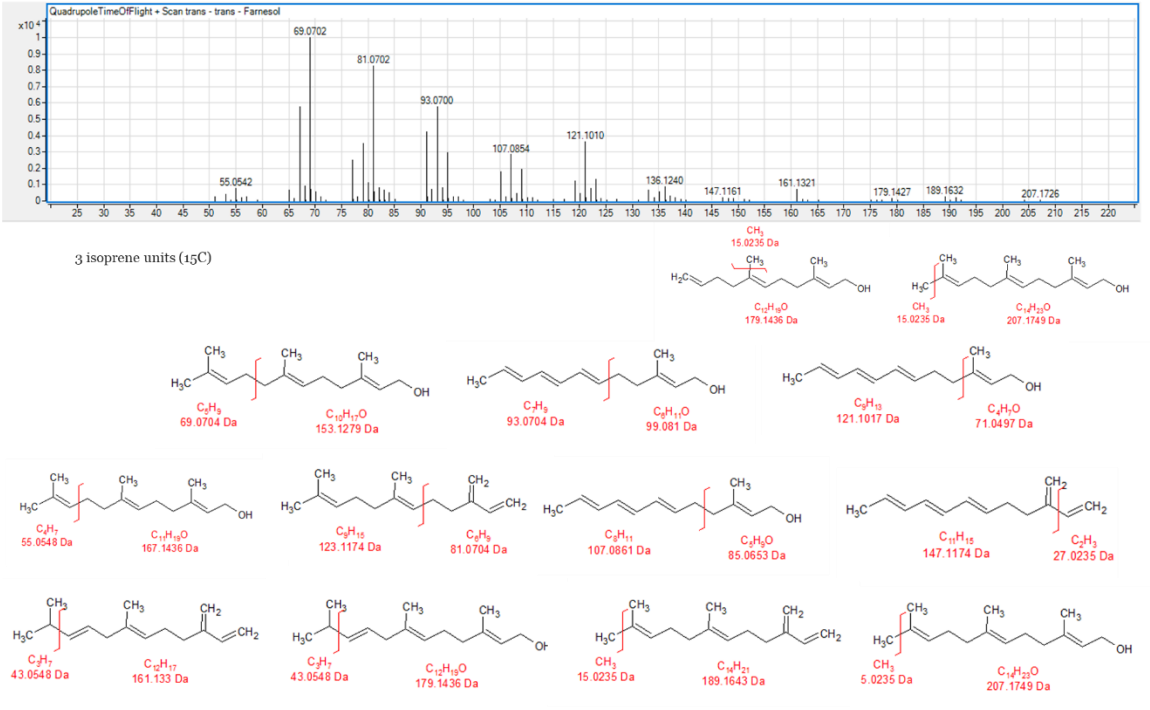
Continuation Supplementary Fig. 5



Continuation Supplementary Fig. 5

trans-trans-Farnesol 222.1984 (C15H26O) RT (41.44 min)

Sesquiterpenoids (acyclic sesquiterpenoids)



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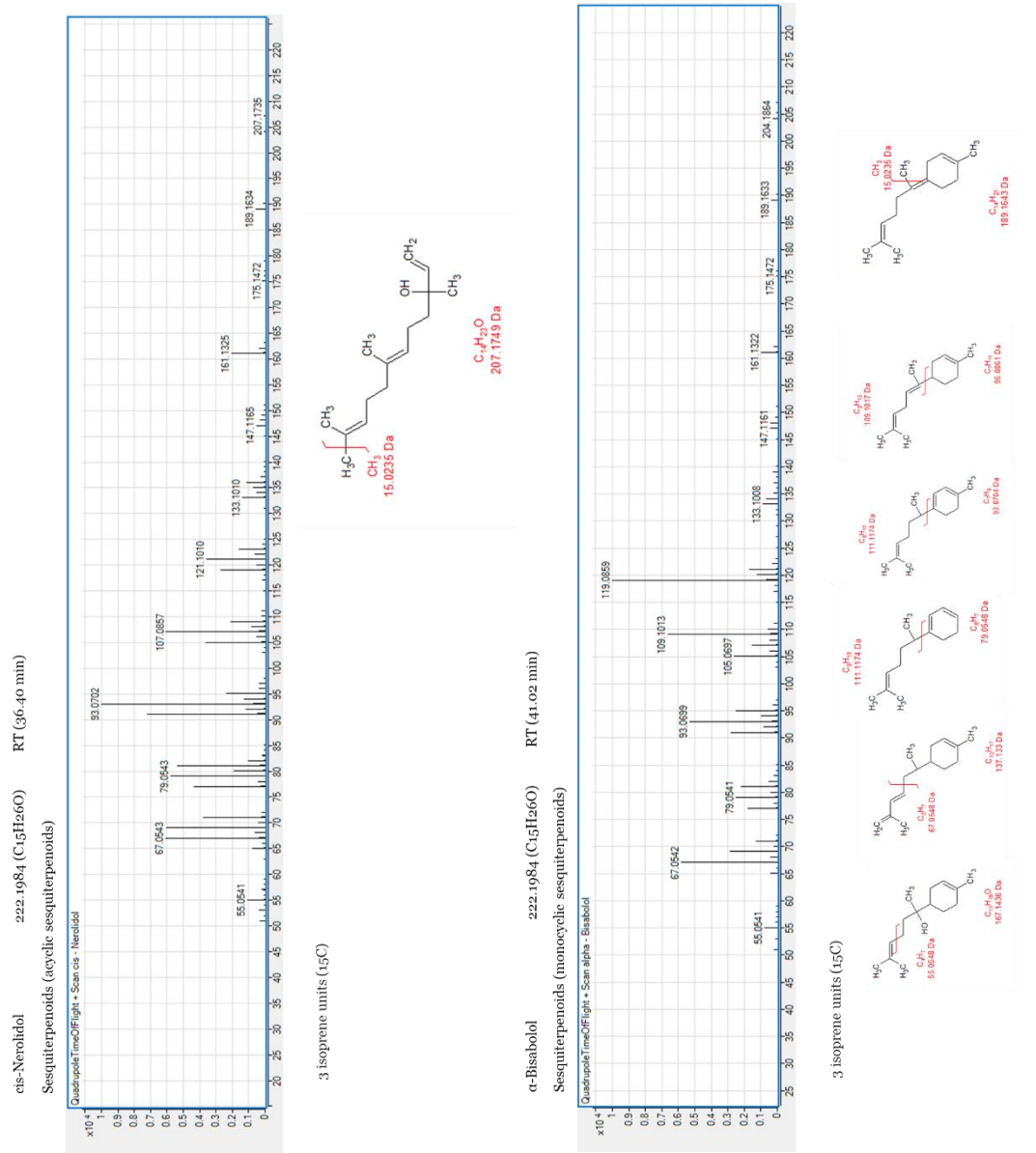
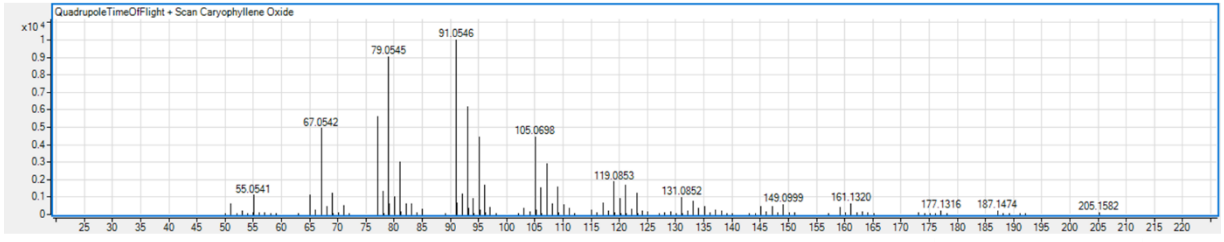


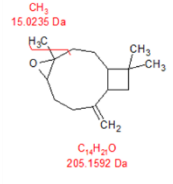
Continuation Supplementary Fig. 5

Caryophyllene oxide 220.1827 ($C_{15}H_{24}O$) RT (38.13 min)

Sesquiterpenoids (monocyclic sesquiterpenoids)

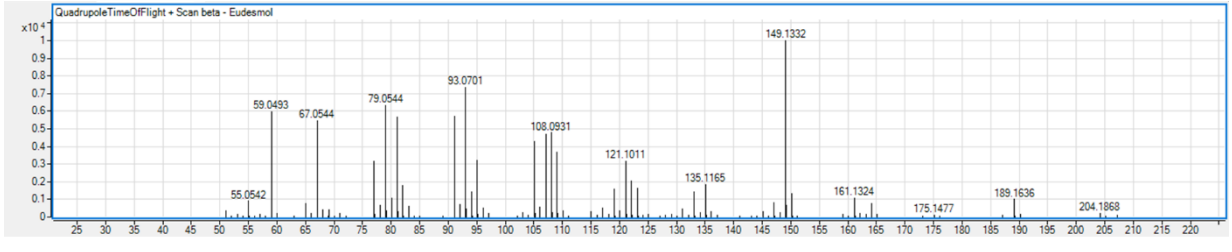


3 isoprene units (15C)

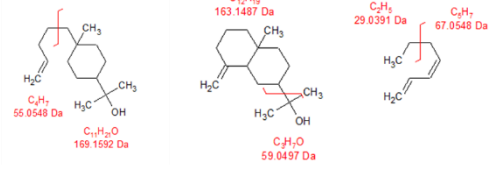


β-Eudesmol 222.1984 ($C_{15}H_{26}O$) RT (40.42 min)

Sesquiterpenoids (bicyclic sesquiterpenoids)



3 isoprene units (15C)



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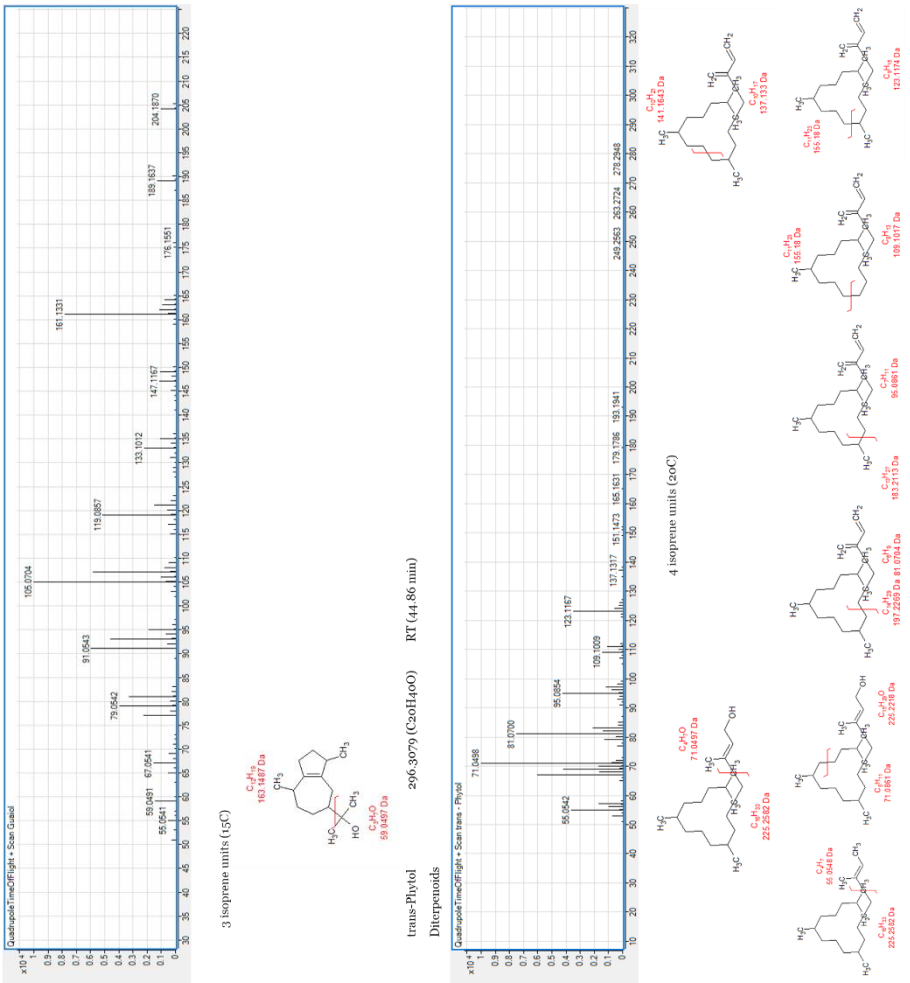
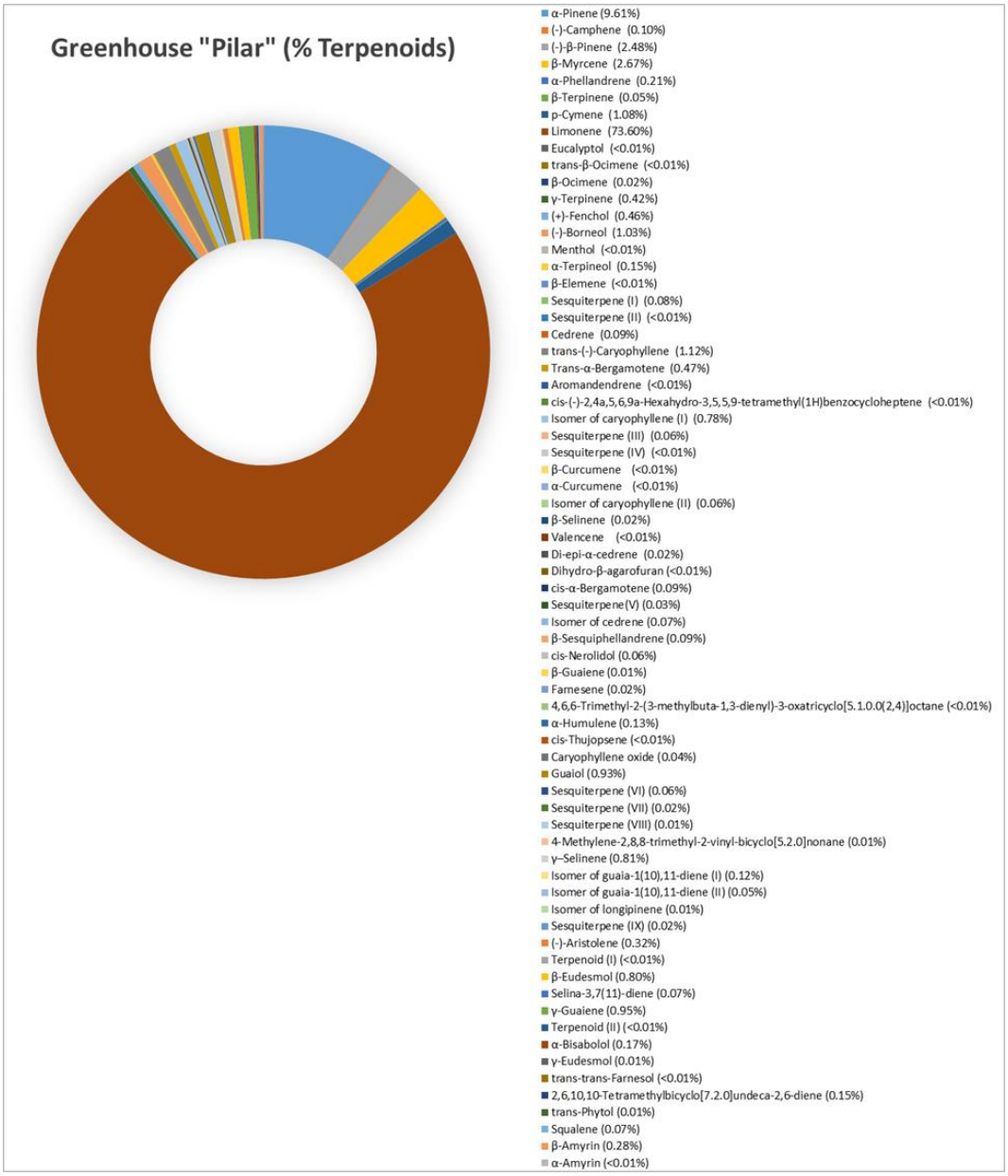
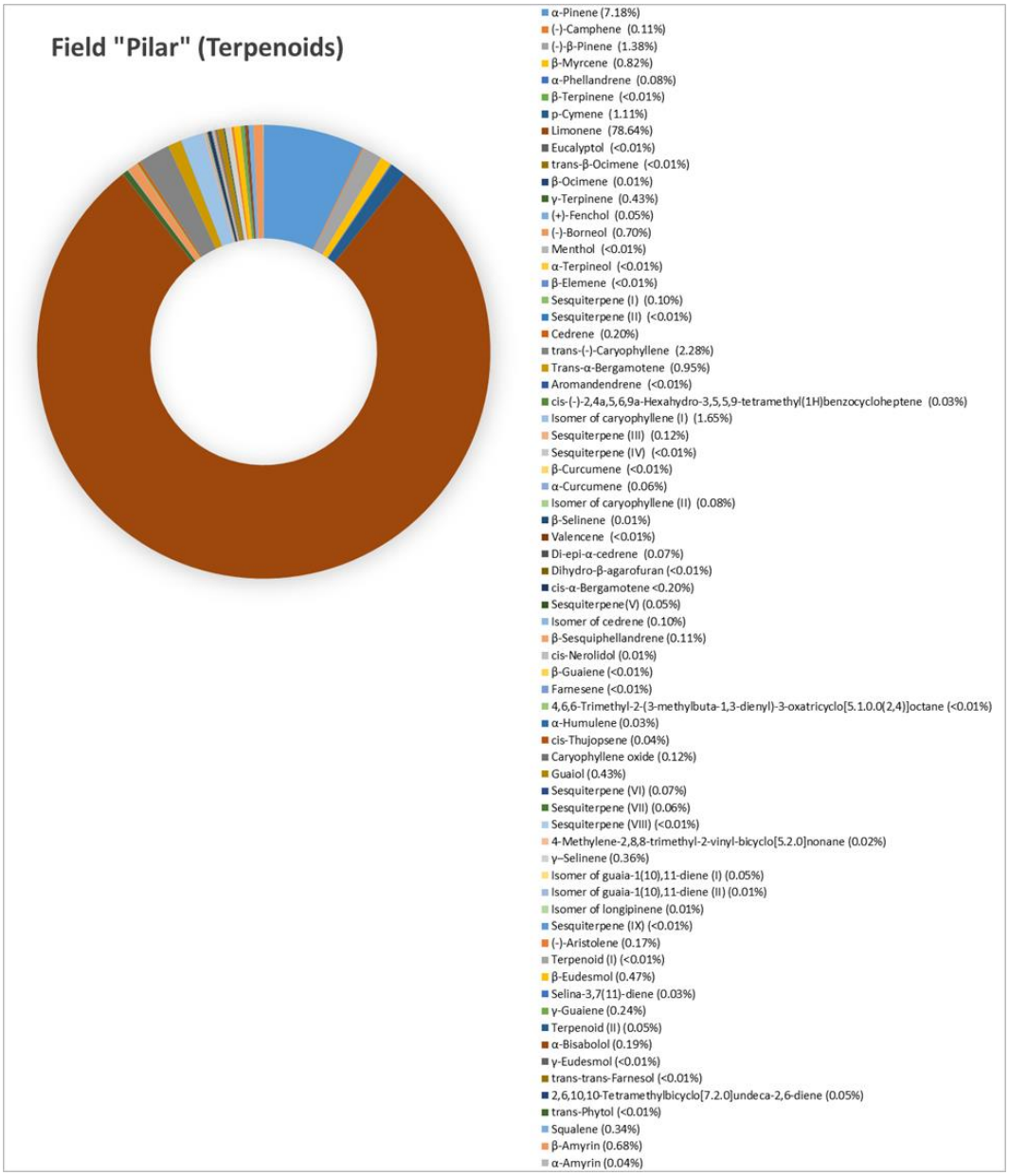




Supplementary Fig. 6. Ring plots comparing the peak area of terpenoids identified in extracts of four varieties ['Pilar' (**A** and **B**), 'Theresa' (**C** and **D**), 'Aida' (**E** and **F**), and 'Juani' (**G** and **H**)] of female *Cannabis* flowers, all cultivated in both field and greenhouse.

Continuation Supplementary Fig. 6



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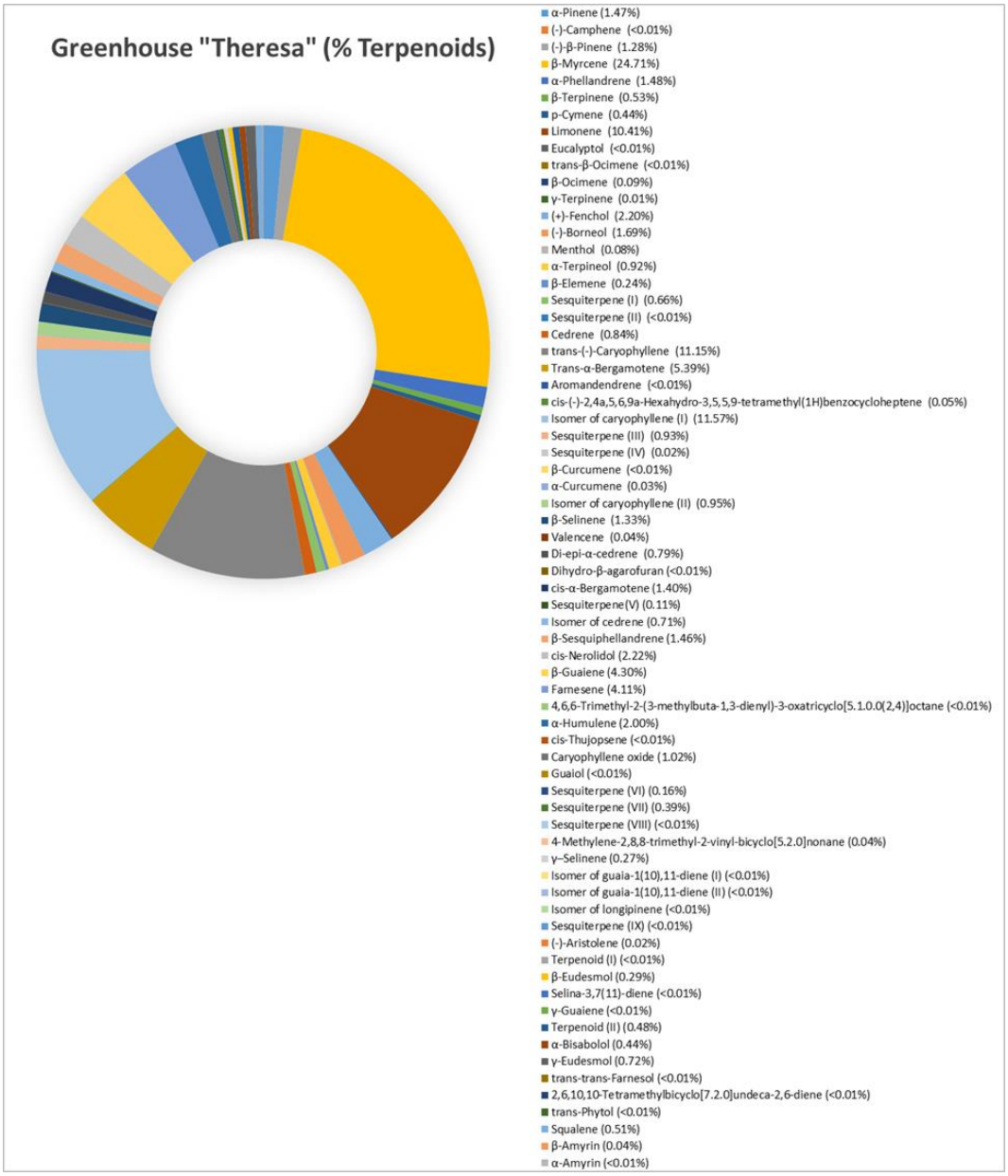

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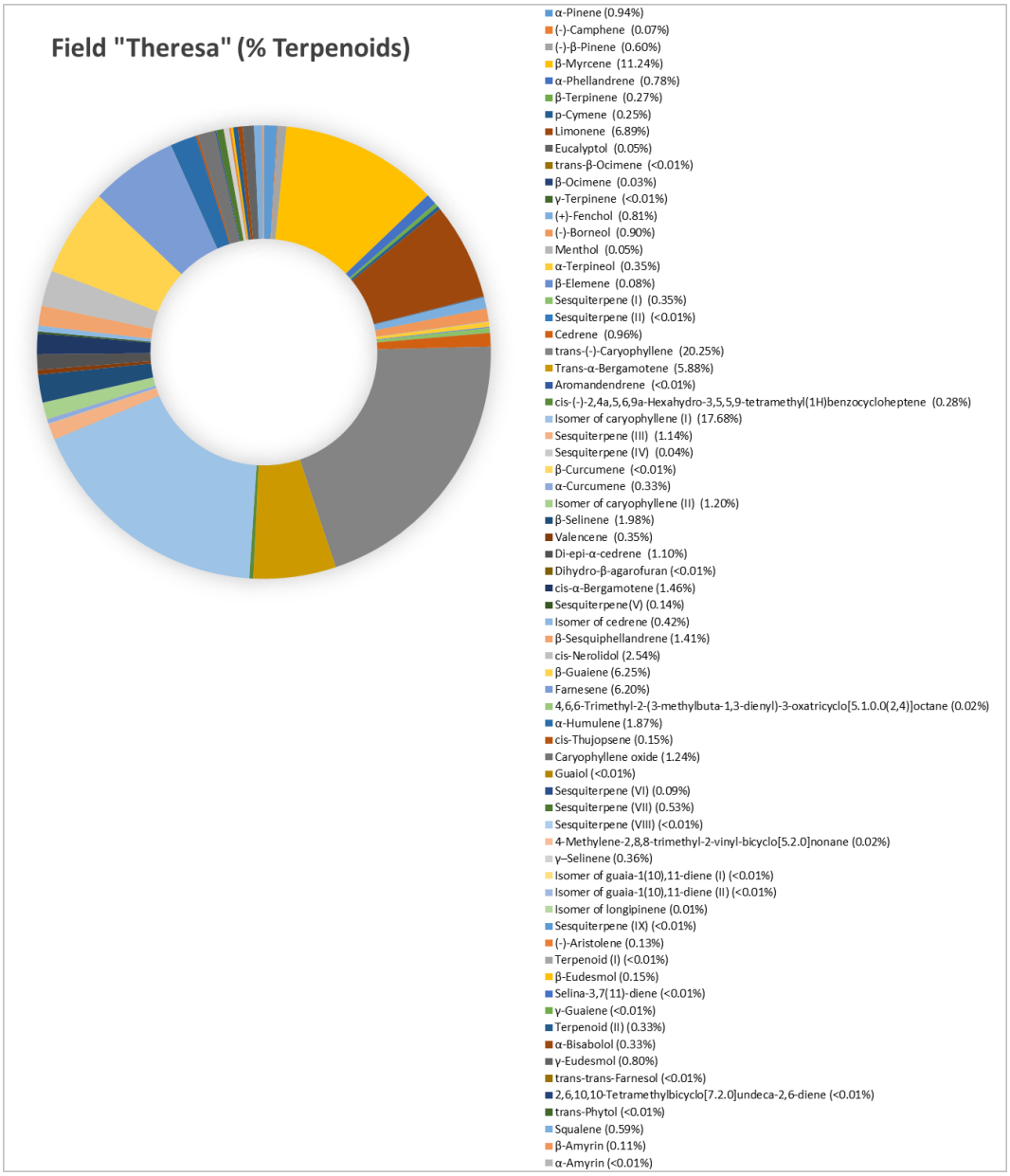

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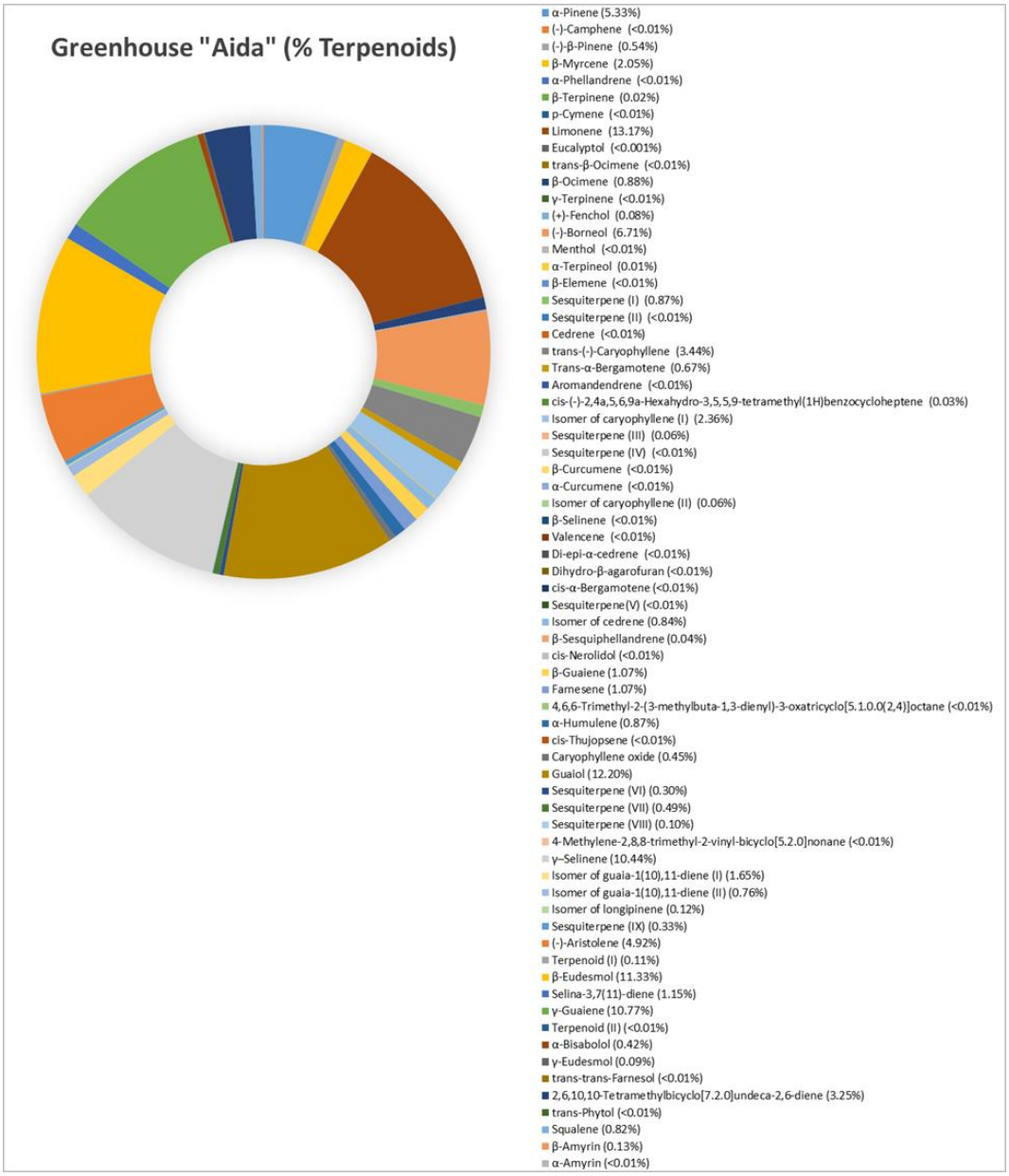

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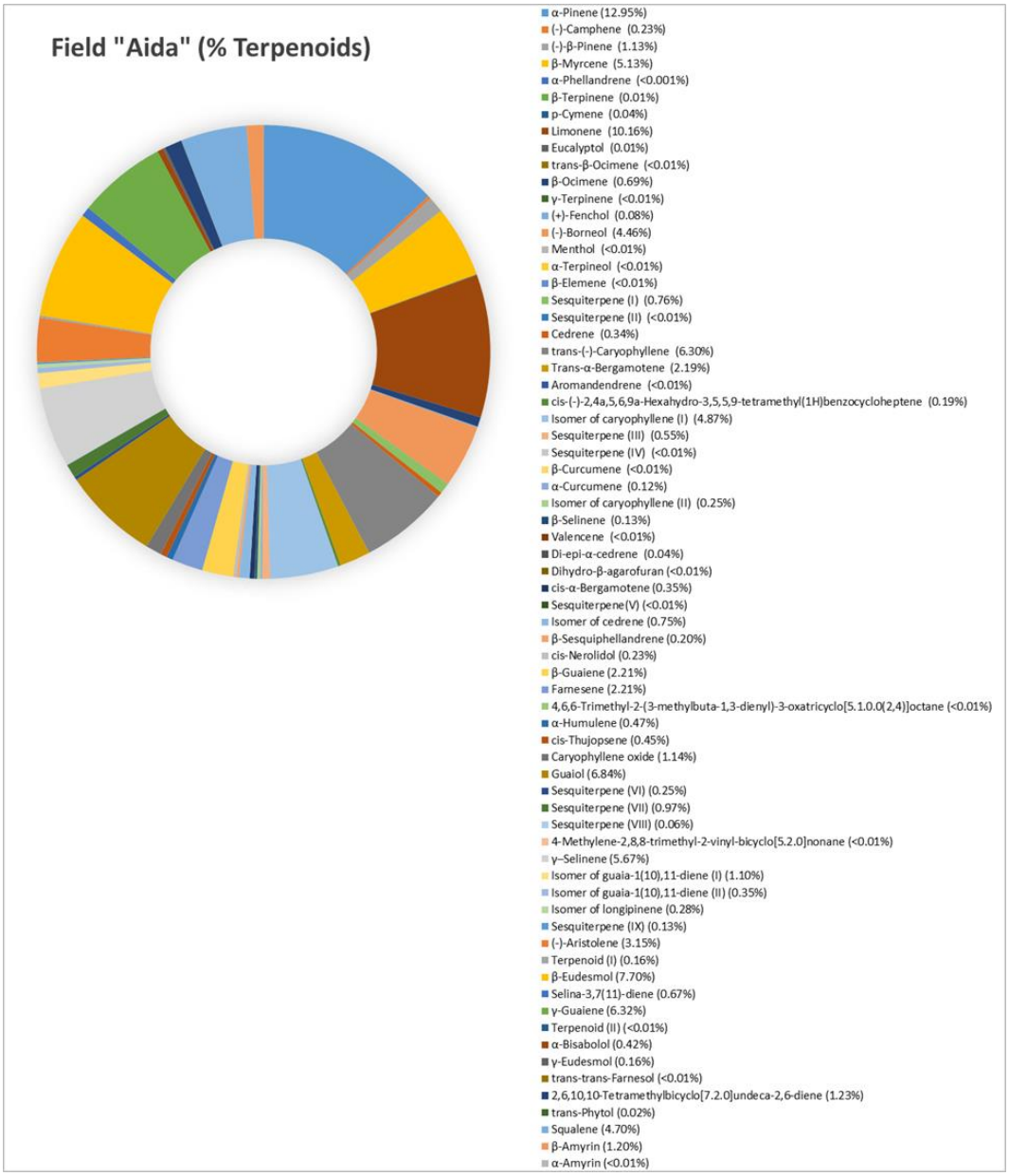

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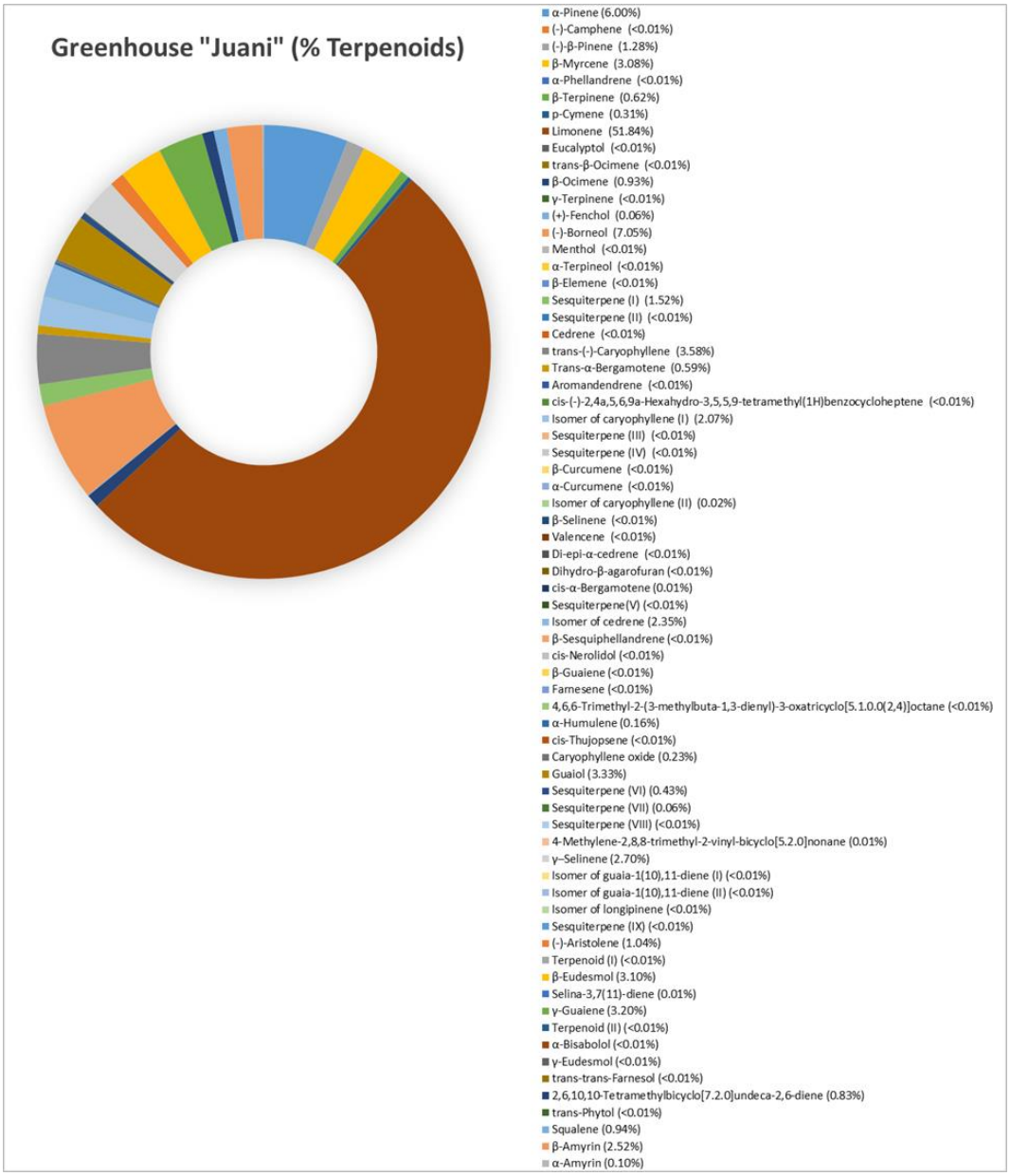

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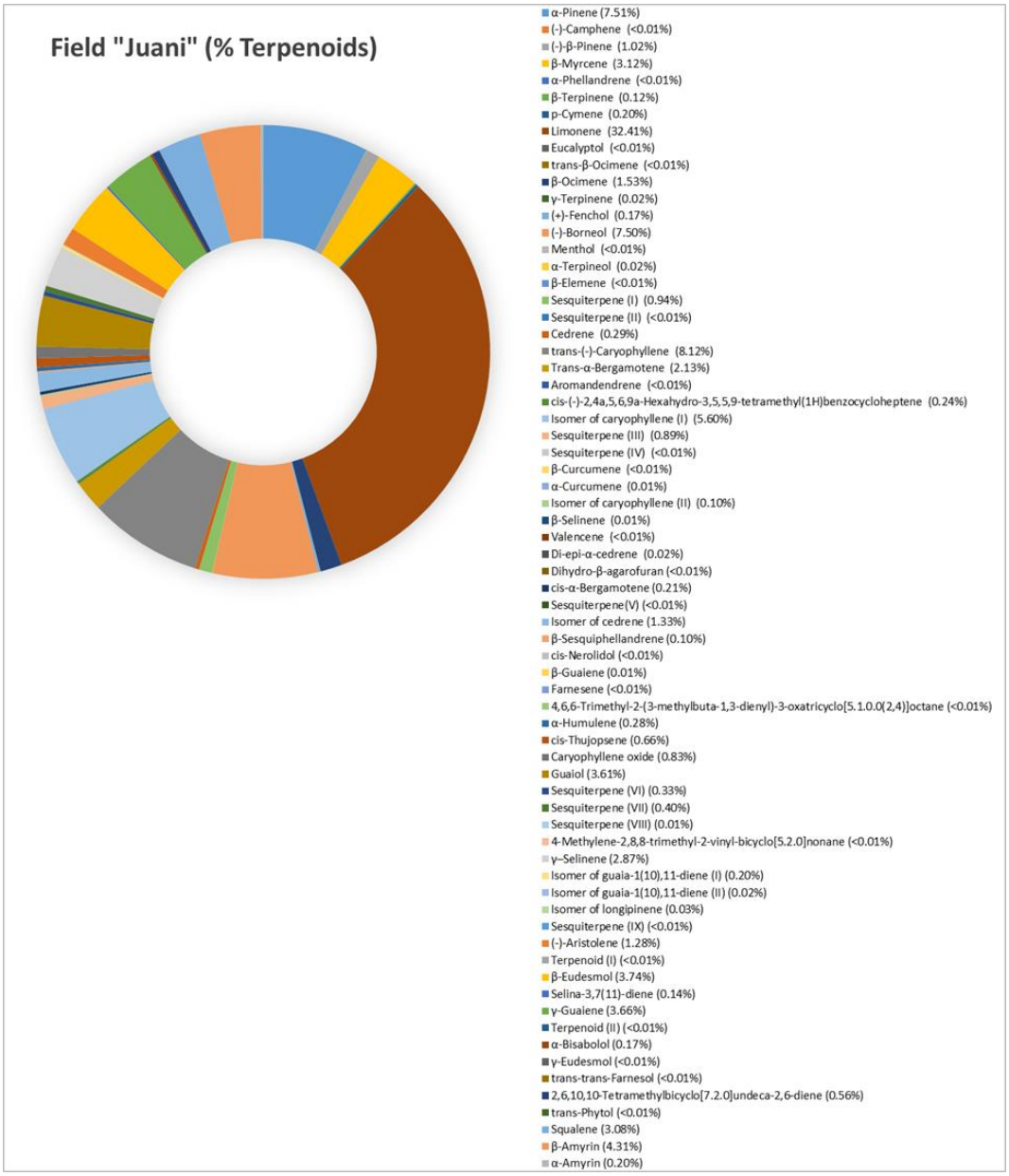

Sudor y metabolómica: Muestreo y preparación de la muestra

Sweat and metabolomics: Sampling and sample preparation

This section encompasses the research dealing with the first steps of the analytical process required by a new clinical sample as sweat: sampling —taking advantage of the multiple forms and situations by which this biofluid can be sampled— and sample preparation —mandatory step to obtain the analytical sample as required for introduction in the target equipment.

Sweat sampling after moderate exercise provided samples from different parts of the body, the composition of which was compared with that of sweat obtained by passive sampling and using different sweat induction procedures. Different sample preparation strategies including derivatization were assayed to obtain a representative snapshot of sweat metabolome, mainly of no polar metabolites as corresponds to the use of GC–MS. Chapter VIII contains this research.

Sampling dry sweat constitutes a novelty; therefore, different solid supports impregnated with different solvents were used as samplers and the composition of the collected samples was compared among them, and also with the composition of fresh sweat. All the samples were analyzed by a dual approach: GC–MS and LC–MS/MS, and the results allowed concluding that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds, as shows Chapter IX.

Amino acids, as key metabolites in the diagnosis and treatment of several diseases, are determined in common biofluids. Quantitative determination of these compounds in sweat have required optimization of the sample preparation step that can consist either of simple dilution or centrifugal solid-phase microextraction, depending on the concentrations of metabolites, prior to introduction into a liquid chromatograph connected to a triple quad mass detector, as shown in Chapter VII.

Chapter VII

Study of sample preparation for quantitative analysis of amino acids in human sweat by liquid chromatography–tandem mass spectrometry

Study of sample preparation for quantitative analysis of amino acids in human sweat by liquid chromatography–tandem mass spectrometry

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Study of sample preparation for quantitative analysis of amino acids in human sweat by liquid chromatography–tandem mass spectrometry

M.M. Delgado-Povedano, M. Calderón-Santiago, F. Priego-Capote, M.D. Luque de Castro

Abstract

The determination of physiological levels of amino acids is important for clinicians to aid in the diagnosis and treatment of several diseases and nutritional status of individuals. Amino acids are frequently determined in typical biofluids such as blood (serum or plasma) and urine; however, there are less common biofluids with other different concentration profiles of amino acids that could be of interest. One of these biofluids is sweat that can be obtained in a no invasive manner and is characterized by low compositional complexity. The analysis of amino acids in human sweat requires the development of sample preparation strategies according to the sample matrix and small collected volume. The influence of sample preparation on the quantitative analysis of amino acids in sweat by LC–MS/MS has been assessed through a comparison between two strategies: dilution of sweat and centrifugal solid-phase microextraction (c-SPμE). In both cases, several dilution factors were assayed for in-depth knowledge of the matrix effects, and the use of c-SPμE provided the best results in terms of accuracy. The behavior of the different amino acids was a function of the dilution factor, thus providing a pattern for sample preparation according to the amino acids to be determined. The concentration of amino acids in sweat ranges between 6.20 ng/mL (for homocysteine) and 259.77 μg/mL (for serine) with precision, expressed as relative standard deviation, within 1.1–21.4%.

Keywords: sweat, amino acid, sample preparation, biomarker, liquid chromatography, mass spectrometry.

1. Introduction

Amino acids, the basic units of proteins, play a key role in the metabolic processes of living organisms. They are the building blocks of peptides and proteins, act as cell signaling molecules, regulate gene expression and critical metabolic pathways, and are key precursors for the synthesis of hormones and low-molecular weight nitrogenous molecules [1].

Determination of physiological levels of amino acids is important to aid in the diagnosis and treatment of hereditary diseases, kidney and liver diseases and neuropathies, but also to evaluate the nutritional status of individuals [1]. Thus, hyperhomocysteinemia has been established as potential biomarkers for neurodegenerative diseases, such as Alzheimer's disease, vascular dementia, cognitive impairment or stroke [2]. Also, the levels of tyrosine, phenylalanine and tryptophan in gastric juice have allowed detection of gastric malignancies [3], or the analysis of branched chain amino acids to discriminate between individuals with and without cardiometabolic risk factors [4].

Derivatization of amino acids has been a common practice prior to their photometric or fluorimetric detection, as most of them do not absorb UV–vis radiation [5]. Among derivatization reagents, *o*-phthaldialdehyde (OPA), phenylisothiocyanate (PITC), 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl-Cl), 1-fluoro-2,4-dinitro-benzene (FDNB) and ninhydrin have been widely used to improve the sensitivity and selectivity for the determination of amino acids [6]. Despite the popularity of these reagents, they suffer from different drawbacks; thus, the instability of OPA derivatives causes irreproducibility in no automated derivatization; Dansyl-Cl based protocols must be developed in the absence of light; FDNB undergoes decomposition in methanol–water solution when exposed to daylight, and PITC is highly toxic [6]. Additionally, derivatization always involves time consuming protocols.

Concerning clinical samples, blood (plasma and serum) and urine have traditionally been used to measure physiological levels of amino acids since both biofluids provide a complete profile of endogenous amino acids [7–9]. For this reason, few methods have been developed to determine these compounds in other clinical samples such as cerebrospinal fluid [10], gastric fluids or sweat, and even less, hair or mammalian tissue [11]. Among these other samples, sweat is especially interesting as it is obtained by no invasive sampling, a key aspect in dealing with people such as hemophiliacs, neonates or

elderly individuals, from whom obtaining blood samples is difficult. Additionally, sweat is a biofluid characterized by a simple matrix composition and, therefore, the probability of interferences from other components is lower than in conventional biofluids [12]. Studies developed by automated analyzers have revealed that the composition of free amino acids in sweat is different from that in other biofluids [13], thus increasing the interest of this sample for quantitative analysis of amino acids for development of diagnostic tools. Methods based on automated analyzers have detected up to 26 amino acids in human sweat, which allows confirming this biofluid as suitable for complete profile of amino acids [13].

To the authors' knowledge, no analytical methods based on LC–MS/MS have been proposed for determination of amino acids in sweat. The objective of this research was to evaluate the influence of sample preparation on quantitative analysis of amino acids in sweat by LC–MS/MS with selected reaction monitoring (SRM) detection mode. For this purpose, two sample preparation approaches were selected: sample dilution to check the influence of matrix effects, and centrifugal solid-phase microextraction (c-SPμE), compatible with the low sample volumes and able to study the clean-up effect. Different sample dilution factors were tested to study the influence of sample composition on the quantitative determination of amino acids.

2. Materials and methods

2.1. Chemicals and reagents

A multistandard solution of amino acids from Sigma–Aldrich (Madrid, Spain) with an individual concentration for each analyte of 0.5 mM±4% in 0.2 N lithium citrate buffer (pH 2.2) with 2% of thiodiglycol and 0.1% of phenol was used to optimize both the chromatographic separation and detection. The multistandard solution contained L-alanine, L-arginine, L-aspartic acid, (±)-carnitine hydrochloride, L-citrulline, L-cysteine, L-glutamic acid, DL-homocysteine, L-isoleucine, L-leucine, DL-lysine, L-methionine, L-ornithine monohydrochloride, D-phenylalanine, DL-proline, L-serine, DL-taurine, L-threonine, L-tyrosine and DL-valine. Additionally, glycine from Merck (Darmstadt, Germany), creatinine and D-tryptophan from Fluka (Madrid, Spain) and L-histidine from

Panreac (Barcelona, Spain) were acquired. A working solution containing 10 μg/mL of each amino acid was prepared in 0.1 M formic acid (FA). All the above solutions were stored at −20 °C until use.

LC–MS grade acetonitrile (ACN) and ammonium formate, ammonium acetate, ammonia, acetic acid and FA from Scharlab (Barcelona, Spain) were used to prepare the chromatographic mobile phases. Deionized water (18 mΩ•cm) from a Millipore Milli-Q water purification system was used to prepare all aqueous solutions. 36.5% (v/v) hydrochloric acid employed for sample preparation was from Panreac.

2.2. Instruments and apparatus

The analyses were performed by RP-LC with MS/MS detection. Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system (consisting of a binary pump, a vacuum degasser, an autosampler and a thermostated column compartment). Detection was carried out by an Agilent 6460 triple quadrupole mass spectrometer equipped with a Jetstream®electrospray ionization (ESI) source. The data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis.

The analytical columns were a 3 μm particle size, 100 mm×4.6 mm i.d. C_{18} Mediterranean Sea from Teknokroma (Barcelona, Spain) and a 3 μm particle size, 100 mm×4.6 mm i.d. Luna hydrophilic interaction chromatography (HILIC) column from Phenomenex (Torrance, CA, USA). Pre-columns, 40 mm×3.0 mm i.d., from Phenomenex were connected to the analytical columns for protection.

A Sorvall Legend Micro 21 microcentrifuge with a standard rotor for 24 tubes with equalized *g*-forces for reproducibility was used for c-μSPE.

2.3. Sweat sampling and storage

The Macroduct® Sweat Analysis System (Wescor, Utah, USA) (Fig. 1) used for sampling consists of a Webster sweat inducer and a Macroduct sweat collector (US Patent 4,542,751). Pilogel®discs (US Patent 4,383,529) (Wescor, Utah, USA) that consist of gel reservoir of pilocarpinium ions were used in the iontophoretic stimulation of sweat

excretion. The sweat inducer provides a current intensity of 1.5 mA for 5 min through two Pilogel discs as electrodes located on the forearm. Previously, the skin was cleaned with ethanol and then with distilled water. The Macroduct device collected sweat for 15 min, which was transferred to plastic microEppendorf tubes and stored at –80 °C until analysis. A total sweat volume of *ca*. 50 µL was collected per individual.

Sweat samples for development and characterization of the method were kindly donated by healthy volunteers at Reina Sofía University Hospital (Córdoba, Spain). Pools of the biofluid used for optimization of the method were prepared by mixing aliquots from all the donors. A number of 14 sweat samples from 8 female and 6 male volunteers were used for quantitative profiling analysis of amino acids. All subjects gave their informed consent for sweat sampling. No restrictions about diet or age were taken into account. All steps from sweat sampling to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki of 2004. The study was approved by the ethical committee of the Reina Sofia University Hospital.

Fig. 1. Macroduct sweat collector.

2.4. Sample preparation

Two different sample preparation protocols were compared in this research. Firstly, aliquots of the sweat pool were 1:1, 1:4, 1:10, 1:20 or 1:50 diluted with 0.1 M FA in water. Then, they were either no spiked or spiked with multistandard solutions at two concentrations per amino acid (50 and 250 ng/mL) and vortexed for homogenization. These samples were analyzed without sample preparation. Secondly, sweat clean-up by c-µSPE using strong cation Micro SpinColumn™ systems (Harvard Apparatus, MA, USA) was applied by following the protocol recommended by the manufacturer for this sorbent material. The protocol used with strong cation Micro SpinColumn™ was as follows:

insertion of 150 µL of water and wait for 10 min for solvation, followed by 150 µL of 50% (v/v) ACN–water for sorbent conditioning, 150 µL of 1% (v/v) FA–water for sorbent equilibration, 75 µL of sample (sweat pool aliquots 1:1, 1:4, 1:10, 1:20, 1:50 and 1:100 diluted with 0.1 M FA), 150 µL of 20% (v/v) ACN–water to wash out potential interferents and, finally, 150 µL of 5% (v/v) ammonia–water for elution of the retained compounds. Centrifugation for 120 s at 1000×*g* was applied after each step, except for elution, which required centrifugation for 240 s at 1000×*g* to complete this step.

2.5. LC–MS/MS analysis

A C_{18} reverse-phase analytical column thermostated at 25 °C was used for chromatographic separation. The mobile phases were water (phase A) and 90% (v/v) ACN–water (phase B) both with 5 mM ammonium formate as ionization agent. The LC pump was programmed with a flow rate of 0.5 mL/min with the following elution gradient: from 2% phase B as initial mobile phase to 5% phase B in 5 min and from 5 to 60% of phase B in 20 min. A post-time of 10 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 5 µL and the injector needle was washed for 12 s between injections with 80% methanol to avoid cross contamination.

MS detection was performed in positive ESI mode at unit resolution in both quadrupoles. The Jetstream ESI source parameters, operating in positive ionization mode, were as follows: nozzle and capillary voltage were set at 0.9 kV and 1 kV, respectively, N_2 nebulizer was flowed at 60 psi, N_2 drying gas flow rate and temperature were 11 L/min and 200 °C, N_2 sheath gas flow rate and temperature were 12 L/min and 400 °C. Detection was carried out by tandem mass spectrometry using the SRM mode. For this purpose, the dwell time was set between 15 and 246.50 ms depending on the transition.

2.6. SRM-based quantitation

Quantitative analysis of amino acids in sweat was carried out using the peak area obtained for the transition of each analyte, which coincided with the chromatographic retention time of the multistandard solution. Calibration models were developed for each analyte using multistandard solutions at nine concentration levels that depended on the

analyte. Five of the calibration points were injected in triplicate to set the confidence intervals.

3. Results and discussion

3.1. Optimization of MS/MS by SRM

Numerous LC–MS/MS methods have been proposed for quantitative analysis of amino acids either using direct analysis or after derivatization to improve sensitivity and selectivity [7,14–18]. The recent advances in both MS instrumentation and sample preparation have allowed developing MS/MS SRM methods with similar or even lower detection limits than those taking benefits after derivatization [19]. For this reason, a strategy without derivatization was adopted for this research.

Optimization of the voltage in the first quadrupole, collision energy and quantitation transition for each amino acid was automatically carried out by direct infusion of standard solutions of the target amino acids at a common concentration of 2 μg/mL. Both positive and negative ionization modes were tested, and the highest sensitivity for all amino acids was achieved by using the positive mode. The most abundant ions of all amino acids under the optimum conditions were selected as precursor ions. As Table 1 shows, the *m/z* values fit the $[M+H]^+$ ion in all instances, except for cysteine, which corresponded to the cystine dimer $[2M+H]^+$.

Isolation and fragmentation of the different amino acids were studied using Q1 voltages from 40 to 200 V and collision energies from 0 to 40 eV. Table 1 lists the results of this study. The Q1 voltage ranged from 50 V for glycine to 140 V for threonine and histidine. On the other hand, the collision energy varied from 5 eV for leucine, glycine and serine up to 30 eV for cysteine and ornithine. In most cases, the product ions were formed by decarboxylation to produce the corresponding immonium ions by loss of 45 Da. In the particular case of lysine, the decarboxylated ion was not stable and tended to loss ammonia with subsequent formation of a product ion at *m/z* 84. The fragmentation pathways for the rest of amino acids involved intramolecular neutral losses or cleavage of the side-chain.

Table 1. Optimization of the MS/MS step for qualitative and quantitative determination of amino acids.

Compound	Retention time (min)	Quantitation transition Precursor ion→ product ion (*m/z*) → (*m/z*)	Q1 voltage[1] (V)	Collision energy (eV)
Glutamic acid	1.9	148 → 84	80	10
Aspartic acid	1.9	134.0 → 87.9	120	10
Glycine	2.2	76.0 → 30.1	50	5
Serine	2.2	106.2 → 60.2	60	5
Taurine	2.2	126.0 → 107.8	100	10
Threonine	2.3	119.7 → 74.0	140	10
Alanine	2.3	90.0 → 44.0	80	20
Citrulline	2.4	176.3 → 69.8	80	10
Cysteine	2.5	241.1 → 152.0	80	30
Proline	2.6	116.0 → 70.1	80	10
Ornithine	2.7	133.0 → 70.0	80	30
Lysine	2.8	147.0 → 84.0	80	10
Valine	2.9	118.1 → 72.0	62	9
Homocysteine	2.9	136.1 → 90.0	68	9
Carnitine	3.0	162.0 → 60.0	120	20
Arginine	3.1	175.0 → 70.1	80	10
Methionine	3.5	149.9 → 103.8	100	10
Creatinine	3.7	114.0 → 86.0	100	10
Leucine/isoleucine	3.9	132.1 → 86.0	60	10
Histidine	4.2	155.9 → 110.0	140	10
Tyrosine	5.1	181.6 → 136.0	80	10
Phenylalanine	8.6	165.9 → 119.9	80	10
Tryptophan	12.8	205.0 → 187.9	80	10

[1]Voltage for filtration of precursor ions.

The study of the parameters involved in the ESI was carried out by testing different ionization agents, particularly, acetic acid, ammonium acetate, ammonium formate or FA. The highest responses were obtained with 5 mM ammonium formate giving a pH of 6.0, at which most amino acids have a neutral net charge. Concerning the ESI, variables such as capillary voltage, sheath gas flow, drying gas flow, gas temperature and nebulizer pressure were studied by a univariate design. The nozzle voltage and sheath temperature were evaluated by a multivariate design involving a 3-level 3^2 factorial design including 9 experiments and 2 central points, which corresponded to star points used to model the

surface. The experiments were carried out by injection of the multistandard solution containing the target amino acids at 1 mg/L. The peak area of the precursor ions from the target amino acids was monitored as response variable. The studied ranges for the nozzle voltage and sheath temperature were 0–600 V and 200–400 ºC, respectively. A positive effect was observed by increasing the sheath temperature, which cannot be tested at values higher than 400 ºC; therefore, it was fixed at this value. The effect of increased nozzle voltage on the signal intensity of the precursor ions was positive, so the range studied for the nozzle voltage was raised up to 2000 V. As no significant improvement of the peak areas was observed between 900 and 2000 V, the nozzle voltage was set at 900 V to avoid source overexertion. The studied ranges for capillary voltage, sheath gas flow, drying gas flow, gas temperature and nebulizer pressure were 500–6000 V, 8–12 L/min, 8–12 L/min, 150–350 ºC, and 30–60 psi, respectively. The effect of the sheath gas flow, drying gas flow and nebulizer pressure on the signal intensity corresponding to the target ions was positive. The sheath gas flow and nebulizer pressure were set at 12 L/min and 60 psi, respectively, and the drying gas flow was established at 11 L/min. The optimized values for capillary voltage and gas temperature were 1000 V and 200 ºC, respectively.

3.2. Optimization of the chromatographic separation step

The commercial multistandard solution containing most of the target amino acids was used to optimize the chromatographic conditions. Two types of columns based on different principles were compared for separation of the target analytes: the HILIC column and the C_{18} column specified under Section 2.

Different chromatographic gradients were tested by using both analytical columns. In the case of HILIC, different gradients based on ACN–water mixtures were evaluated, and the best separation was achieved with a gradient from 90% ACN in water acidified with 0.1% (v/v) acetic acid (phase A) to 40% (v/v) ACN–water with 5 mM ammonium acetate (phase B). The optimum gradient was as follows: phase A for 5 min, after which a linear gradient for 18 min was programmed up to 80 phase B. Then, a second ramp up to 100% B for 5 min was applied. On the other hand, combinations of water and ACN using 5 mM ammonium formate as ionizing agent were assayed in reverse-phase chromatography with the C_{18} column. The best results were obtained with 5 mM of ammonium formate in 100%

water (phase A) and 90% (v/v) ACN–water solutions (phase B) with the gradient described under Section 2.

The pH of the mobile phases strongly affected the chromatographic separation in both chromatographic modes. Thus, pHs between 3.2 and 7.0 were evaluated, being pHs 5.5 and 6.0 the best to achieve a suited analytes–chromatographic sorbent interaction in HILIC and RP chromatography, respectively; while pHs out of the 5.0–7.0 range led to irreproducible results owing to inefficient retention. Fig. 2 shows the chromatographic profile obtained with the two columns and ten representative amino acids eluting within the chromatographic gradient.

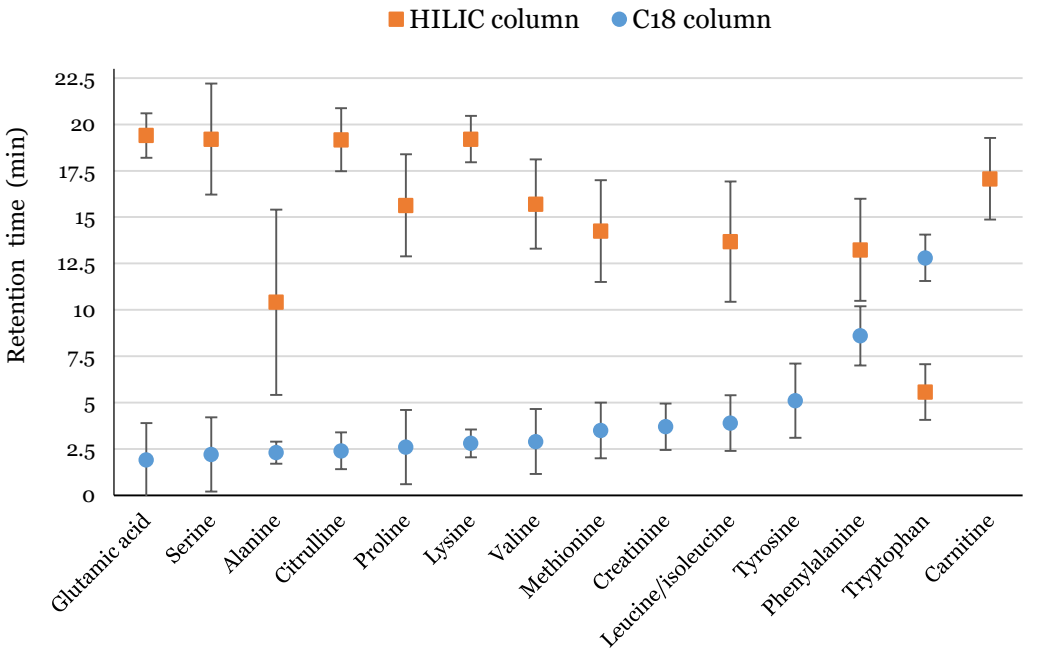

Fig. 2. Retention times and peak widths of the target compounds obtained by the HILIC column as compared to those provided by the C_{18}RP column (for details, see text).

As can be seen, a better separation was obtained using the HILIC column since all the amino acids were eluted within the 10–20 min interval, as compared to RP chromatography in which elution was completed within the 2.0–8.6 min interval, except

for tryptophan that eluted at 12.8 min. However, the best resolution, expressed as the width of the chromatographic peaks, was obtained by the C_{18} column since the HILIC column provided wider peaks. This criterion relates a better resolution with a better sensitivity, which justified selection of the C_{18} column. Because leucine and isoleucine were not properly separated owing to their isomeric character, they were quantified together. Supplementary Fig. 1 shows the SRM chromatograms from a multistandard solution and using the two chromatographic methods.

3.3. Calibration models

The calibration curve for each analyte was run by using the peak areas of the selected transition obtained at different dilutions (with 0.1 M FA in water) of the stock solution containing all the amino acids. The linear dynamic ranges and regression coefficients are shown in Table 2, with values for the latter above 0.98 for all analytes, and upper limit for calibration dependent on the target compound. Thus, some amino acids fitted linearly up to concentrations *ca*. 1.25 µg/mL, while others only reached 25 ng/mL.

The limits of detection (LOD) and quantitation (LOQ) of the method for each amino acid were calculated as the concentration providing signals three and ten times, respectively, higher than the background noise at elution times close to each peak; and they were between 0.08–1.50 ng/mL and 0.25–5 ng/mL, respectively, for all analytes. The individual values in all cases are shown in Table 3.

Once the calibration models were obtained, the influence of the two sample preparation alternatives, dilution and c-SPµE, on the quantitative determination of amino acids in sweat was tested.

3.4. Dilution of sweat as sample preparation step prior to quantitative analysis of amino acids

Dilution of sweat by using 0.1 M FA in water was evaluated for analysis of amino acids without any other sample preparation step. For this purpose, the dilution factors 1:1, 1:4, 1:10, 1:20 and 1:50 were tested to know the influence of matrix effects on the accuracy of the method. Three sweat aliquots of the prepared pool were diluted in the FA aqueous medium at each dilution factor. One of them was directly used for quantitative analysis of

the amino acids in sweat, while the other two aliquots were spiked with all target amino acids at 50 or 250 ng/mL. Each aliquot was analyzed in triplicate.

Table 2. Linear calibration ranges, calibration equations and regression coefficients for the target amino acids.

Compound	Calibration curve	Linear calibration range (ng/mL)	Regression coefficient
Glutamic acid	y = 164.6x+2785	0.25–625	0.9930
Aspartic acid	y = 10.09x+39.47	0.25–50	0.9800
Glycine	y = 14.04x+102.6	0.25–50	0.9830
Serine	y = 92.07x+908.6	0.25–50	0.9980
Taurine	y = 0.59x+10.09	5–250	0.9970
Threonine	y = 0.677x+47.66	0.25–625	0.9800
Alanine	y = 57.96x+277.1	0.25–50	0.9910
Citrulline	y = 185.53x+844.86	0.25–50	0.9864
Cysteine	y = 9.9656x+4.2383	0.25–50	0.9973
Proline	y = 1382x+5380	0.25–50	0.9920
Ornithine	y = 167.6x+2440	0.25–250	0.9860
Lysine	y = 416.8x+556.2	0.25–25	0.9900
Valine	y = 103x+482.9	0.25–1250	0.9940
Homocysteine	y = 2.983x+6.392	2.5–50	0.9932
Carnitine	y = 1636.5x+589.37	0.25–50	0.9984
Arginine	y = 246.44x+1814.1	0.25–50	0.9863
Methionine	y = 24.55x+138.1	0.25–625	0.9970
Creatinine	y = 187.3x+110.4	0.25–25	0.9890
Leucine/isoleucine	y = 642.5x+782.9	0.25–50	0.9970
Histidine	y = 62.79x+10718	0.25–1250	0.9870
Tyrosine	y = 98.269x+795.33	0.25–250	0.9908
Phenylalanine	y = 343.5x+3028.2	0.25–1250	0.9988
Tryptophan	y = 89.19x+696.67	0.25–625	0.9984

y: peak area; x: concentration as ng/mL.

Table 3. Limits of detection and quantitation for the target amino acids.

Compound	**LOD (ng/mL)**	**LOQ (ng/mL)**
Glutamic acid	0.08	0.25
Aspartic acid	0.08	0.25
Glycine	0.08	0.25
Serine	0.08	0.25
Taurine	1.50	5.00
Threonine	0.08	0.25
Alanine	0.08	0.25
Citrulline	0.08	0.25
Cysteine	0.08	0.25
Proline	0.08	0.25
Ornithine	0.08	0.25
Lysine	0.08	0.25
Valine	0.08	0.25
Homocysteine	0.75	2.50
Carnitine	0.08	0.25
Arginine	0.08	0.25
Methionine	0.08	0.25
Creatinine	0.08	0.25
Leucine/isoleucine	0.08	0.25
Histidine	0.08	0.25
Tyrosine	0.08	0.25
Phenylalanine	0.08	0.25
Tryptophan	0.08	0.25

The accuracy values, estimated as mean percentage of the three analyses per aliquot, are listed in Table 4 for each spiked concentration. Only values within the 75–125% range are included in Table 4, assuming a maximum variability of 25% for the analysis of amino acids in sweat. The best accuracy values at the two spiked concentrations were provided by the 1:20 and 1:50 dilution factors. Thus, 9 and 11 amino acids out of 23 were properly quantified (assuming 25% variability in accuracy terms) by the 1:20 and 1:50

dilution factors, respectively, at the spiked level of 50 ng/mL. Nevertheless, when the spiked concentration was 250 ng/mL the number of properly quantified amino acids decreased to 6 and 8 for dilution factors 1:20 and 1:50, respectively.

Only 10 out of the 23 amino acids reported values within the preset interval for the two spiked concentrations at one among the five assayed dilution factors. The low accuracy for the rest of amino acids (*viz.*, cysteine, homocysteine, aspartic acid, ornithine, taurine, threonine, valine, lysine, methionine, tyrosine, phenylalanine and leucine/isoleucine) could be attributed to significant matrix effects, which are not suppressed by sample dilution. Despite quantitative analysis of the complete set of amino acid is not possible by direct injection into the chromatograph of diluted sweat, several dilution factors should be applied to maximize the number of amino acids to be quantified. Taking into account the matrix effects, other sample preparation protocol based on c-SPμE was tested to minimize or suppress them.

3.5. Sample preparation based on c-SPμE prior to quantitative analysis of amino acids in sweat

Centrifugal SPμE was assayed as sample preparation step to overcome sweat matrix effects. As amino acids can be positively or negatively charged depending on the pH of the solution, strong ionic sorbents should be an appropriate material for selective retention of the analytes. Two types of strong sorbents were tested: cation and anion resins.

Preliminary experiments revealed that retention of amino acids on the cationic sorbent was more efficient than on the anionic, since the sorbent–analyte interaction is stronger in 0.1 M HCl medium (solution used for retention of amino acids in the cationic resin) than in 5% (v/v) ammonia in water (used for retention of amino acids in the anionic resin). For this reason, the strong cationic sorbent was selected for evaluation of c-SPμE in the analysis of amino acids in sweat. Supplementary Fig. 2 shows a chromatogram from sweat spiked with 50 ng/mL of each amino acid by using c-SPμE as sample preparation strategy.

As in the previous study, different dilution factors were tested to know their influence of sweat matrix effects. Initially, no spiked aliquots (three per experimental condition) of sweat were 1:1, 1:4, 1:10, 1:20, 1:50 and 1:100 (v/v) diluted with 0.1 M HCl in water.

Table 4. Accuracy estimated in the analysis of diluted sweat. Recovery values are expressed as percent for each amino acid as a function of the dilution factor and the spiked concentration. Values below 75% or above 125% are not shown.

	Dilution									
	1:1		**1:4**		**1:10**		**1:20**		**1:50**	
Compound\spiked concentration (ng/mL)	**50**	**250**	**50**	**250**	**50**	**250**	**50**	**250**	**50**	**250**
Glutamic acid	–	–	–	–	83.8 ±4.3	–	114.3 ±8.5	93.1± 2.2	–	94.8 ±3.2
Aspartic acid	–	–	–	–	–	–	–	105.8 ±2.4	–	–
Taurine	–	–	–	–	–	–	–	–	84.5± 9.4	–
Serine	103.8 ±5.4	101.7 ±4.1	97.1± 2.0	86.9 ±1.8	104.0 ±6.0	116.2± 5.3	107.2 ±3.4	102.4 ±2.2	116.9 ±7.2	97.3± 5.8
Glycine	93.2± 5.9	90.5± 4.6	81.9± 2.1	–	75.6± 2.1	–	79.7± 1.8	–	80.8± 2.5	–
Threonine	–	–	–	–	–	–	116.9 ±20.0	–	83.8± 3.5	–
Alanine	89.4± 5.8	87.0± 6.5	94.3± 6.5	–	85.1± 11.8	–	–	116.9 ±5.5	–	122.6 ±4.7
Citrulline	–	–	107.7 ±60.9	–	121.4 ±563	121.1± 35.3	99.5± 11.8	–	79.4± 4.0	–
Cysteine	–	–	–	–	–	–	–	–	–	–
Proline	95.7± 17.8	93.6± 14.8	85.8± 4.6	–	81.6± 3.8	–	94.8± 1.0	–	111.8± 10.0	–
Ornithine	–	–	–	–	–	80.5± 253.6	–	–	–	–
Lysine	–	–	–	–	–	–	105.4 ±0.6	–	106.2 ±9.7	–
Homocysteine	–	–	–	–	–	–	–	–	–	–
Valine	–	–	–	–	–	–	–	–	76.4± 6.9	–
Carnitine	–	–	89.5± 79.5	76.5± 13.0	–	–	–	–	–	–
Arginine	–	–	–	–	98.0 ±7.7	–	112.3 ±4.9	–	113.6 ±8.6	112.3 ±2.9
Methionine	–	–	–	–	–	–	–	–	119.2 ±20.1	–
Creatinine	–	–	89.0± 31.8	81.8 ±31.2	84.0 ±4.6	–	–	–	–	–
Leucine/isoleucine	–	–	–	–	–	82.6± 9.0	–	–	–	–
Histidine	94.0± 49.3	88.8± 40.0	–	–	–	–	124.2 ±5.9	119.0 ±8.9	109.6 ±12.5	104.5 ±0.6
Tyrosine	–	–	–	–	–	–	–	78.8 ±6.3	–	103.3 ±4.7
Phenylalanine	–	–	–	–	–	–	–	–	–	95.6± 2.9
Tryptophan	–	–	92.4± 1.9	–	79.1± 14.7	–	–	–	–	110.5 ±7.6

For most of the amino acids the dilution factors providing the maximum concentrations were 1:20 and 1:50 (v/v) (exceptionally, cysteine was optimally quantified

at 1:100 dilution factor; arginine, histidine, homocysteine and aspartic acid at 1:10 dilution factor; and glutamic acid at 1:4 dilution factor). Attending to the behavior of the majority of the amino acids, two dilution factors, 1:20 and 1:50, were selected to estimate the accuracy of the method by using c-SPμE by spiking sweat aliquots with the target analytes at 50 and 250 ng/mL. The aliquots were then subjected to SPE and analyzed by LC–MS/MS, and the accuracy values obtained were listed in Table 5 after discarding values of variability above 20% (*viz*., out of the range from 80–120%).

For the 1:20 dilution factor, 13 amino acids provided accuracy values within the established limits at both concentration levels, while other five amino acids fulfilled the accuracy restriction only at 50 ng/mL. Despite of the fact that 1:20 was not the optimum dilution factor for alanine, citrulline, cysteine, aspartic acid, proline, lysine, valine, carnitine, methionine, phenylalanine, tryptophan and leucine/isoleucine, acceptable accuracy values were obtained for all them at both concentration levels.

Concerning the 1:50 dilution factor, only 7 amino acids provided acceptable accuracy values at both spiked concentrations, while other 6 amino acids led to acceptable accuracy values at one spiked level. One amino acid, tyrosine, that did not provide accuracy values within the preset interval at 1:20 dilution factor was properly quantified at 1:50 dilution.

It can be summarized that amino acids such as cysteine, aspartic acid, lysine, valine, methionine, leucine/isoleucine, tyrosine and phenylalanine at the two spiked levels, and taurine and threonine at one spiked level that were not determined accurately by direct analysis of diluted sweat provided good accuracy values by c-SPμE. Therefore, the implementation of c-SPμE allowed reducing or suppressing matrix effects of sweat and made possible to obtain better accuracy values. This behavior is explained by the double clean-up effect of the c-SPμE procedure: in the sample loading by no retention of interferents, and in the washing step by removal of interferents by a suited washing solvent composition. On the other hand, other amino acids such as homocysteine and ornithine did not provide acceptable accuracy by any of the two sample preparation strategies, which could be explained by losses in the different steps of the SPE protocol.

Table 5 also contains information on the precision of the method since each accuracy value was estimated by three sweat aliquots. As can be seen, the repeatability, estimated as relative standard deviation, ranged from 1.1 to 21.4% and from 0.5 to 15.1% for 1:20 and 1:50 dilution factors, respectively.

Table 5. Accuracy estimated in the study of centrifugal SPμE for 1:20 and 1:50 diluted sweat. Recovery values are expressed as percent for each amino acid as a function of the dilution factor and the spiked concentration. Values below 80% or above 120% are not shown.

	Dilution			
	1:20		**1:50**	
Compound\spiked concentration (ng/mL)	**50**	**250**	**50**	**250**
Glutamic acid	96.5±4.7	100.1±4.3	91,88±7,18	107,86±2,88
Aspartic acid	97.1±5.2	98.3±11.1	–	–
Taurine	120.7±15.7	–	112,22±11,26	–
Serine	99.9±4.1	89.2±7.6	104,13±0,45	88,53±1,38
Glycine	86.6±3.9	–	94,31±3,32	–
Threonine	–	–	–	111,67±15,07
Alanine	93.6±11.2	90.9±7.6	85,23±5,27	86,51±8,91
Citrulline	84.0±2.4	98.0±2.4	112,34±4,39	–
Cysteine	107.4±4.6	87.1±8.8	–	–
Proline	100.7±5.1	112.7±0.8	117,65±6,68	–
Ornithine	–	–	–	–
Lysine	85.3±2.4	89.78±0.9	114,19±2,84	115,95±8,47
Homocysteine	–	–	–	–
Valine	109.1±10.3	111.2±21.4	–	–
Carnitine	96.7±1.1	116.8±1.6	–	–
Arginine	–	–	–	–
Methionine	102.1±5.9	104.9±5.6	–	89,69±5,27
Creatinine	93.9±2.0	–	–	–
Leucine/isoleucine	93.4±9.8	98.0±6.4	93,80±10,86	105,62±4,07
Histidine	–	–	–	–
Tyrosine	82.6±15.5	–	82,81±4,99	85,52±8,97
Phenylalanine	86.2±5.8	93.7±6.8	102,71±1,86	94,44±3,48
Tryptophan	–	119.0±9.4	–	–

3.6. Analysis of women and men sweat samples

The method based on c-SPμE and dilution was used to analyze sweat samples of female and male volunteers. After analysis of all samples, the concentration of each amino acid in sweat was calculated. Fig. 3 shows the average concentration of each amino acid in 8 sweat samples from women (Fig. 3A) and in 6 sweat samples from men (Fig. 3B). These results show that the average concentration of all amino acids in sweat was higher in women than in men. Concerning the profile of amino acids, the most concentrated amino acids in male and female individuals were serine, histidine, glycine, alanine, glutamic acid and valine. Additionally, aspartic acid was also found at higher concentrations in female than in male volunteers. It is also worth mentioning that the variability in concentrations of amino acids was particularly low, except for arginine that reported a high variability in the studied individuals.

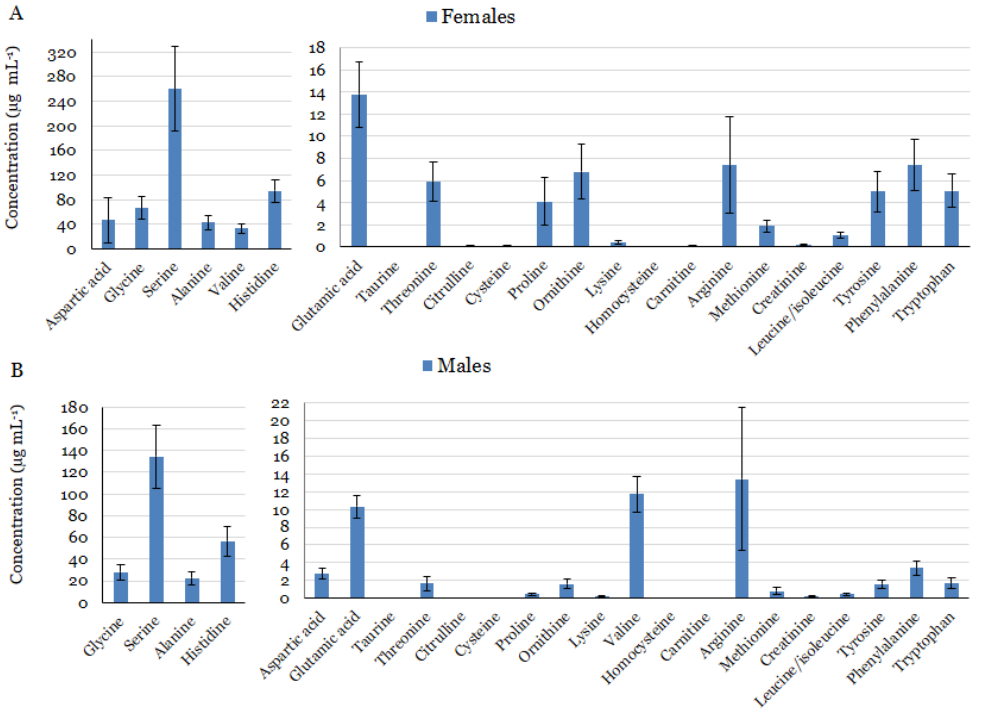

Fig. 3. Quantitative profiling of amino acids in 8 sweat samples from women (A) and in 6 sweat samples from men (B).

4. Conclusions

The search for biofluids complementary to blood (serum or plasma) and urine is one of the present trends in clinical metabolomics analysis. In this research, the potential of sweat for quantitative profiling of amino acids has been evaluated with an in-depth study of sample preparation approaches, being c-SPμE the preferred option in terms of accuracy. The developed research has provided the following conclusions: (i) sweat can be an excellent sample for quantitative profiling analysis of amino acids in clinical studies, which is of interest taking into account the biomarker role of amino acids; (ii) the chromatographic resolution in the separation of amino acids is better with a C_{18} analytical column than with a HILIC phase; (iii) the small volume of sweat collected per individual makes mandatory the use of microformat sample preparation approaches, being c-μSPE an excellent option; (iv) dilution of sweat is mandatory to decrease matrix effects and make possible absolute quantitation of amino acids; (v) amino acids behave differently; therefore, compromise solutions are necessary for their determination in a single analysis.

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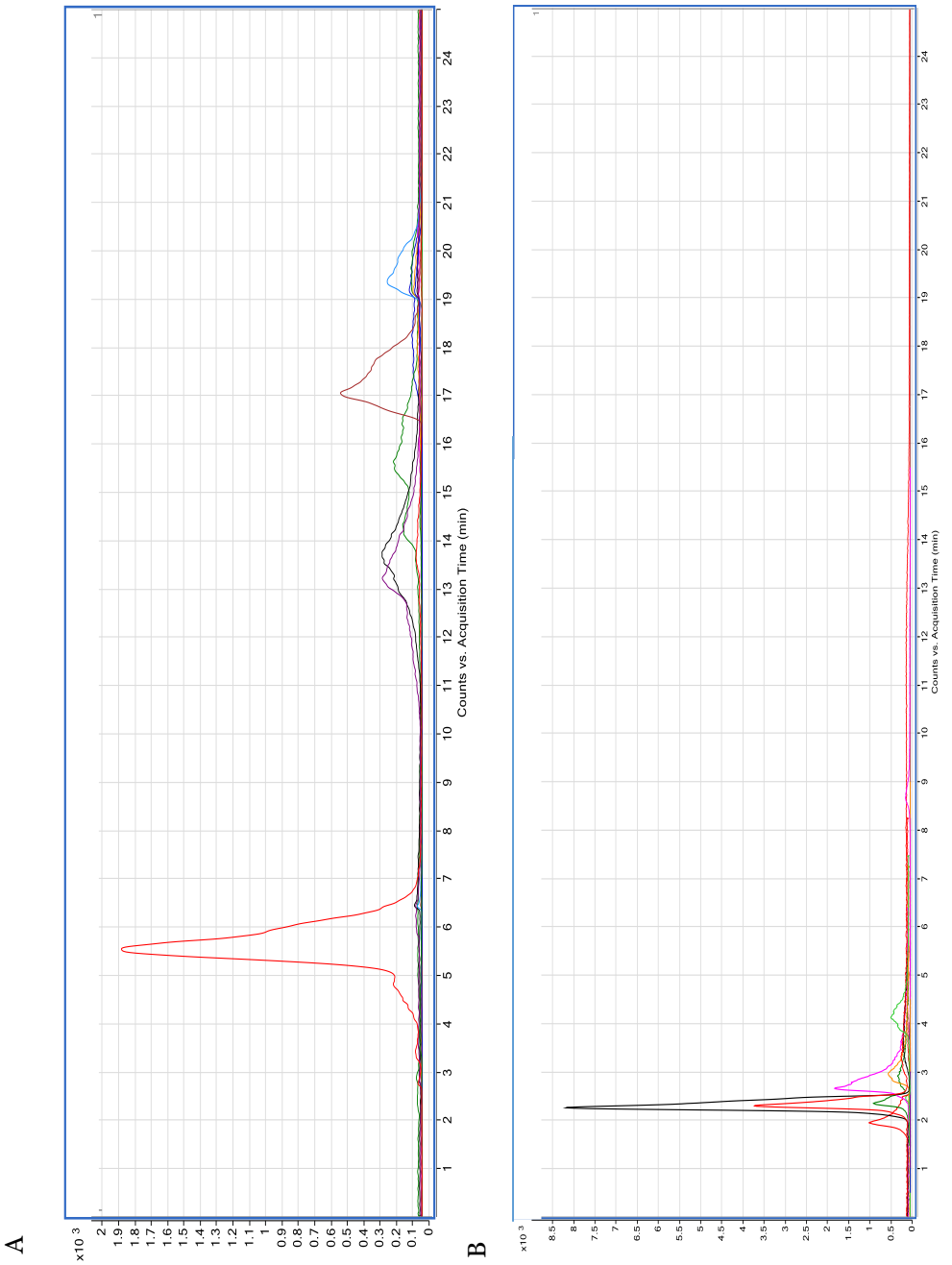
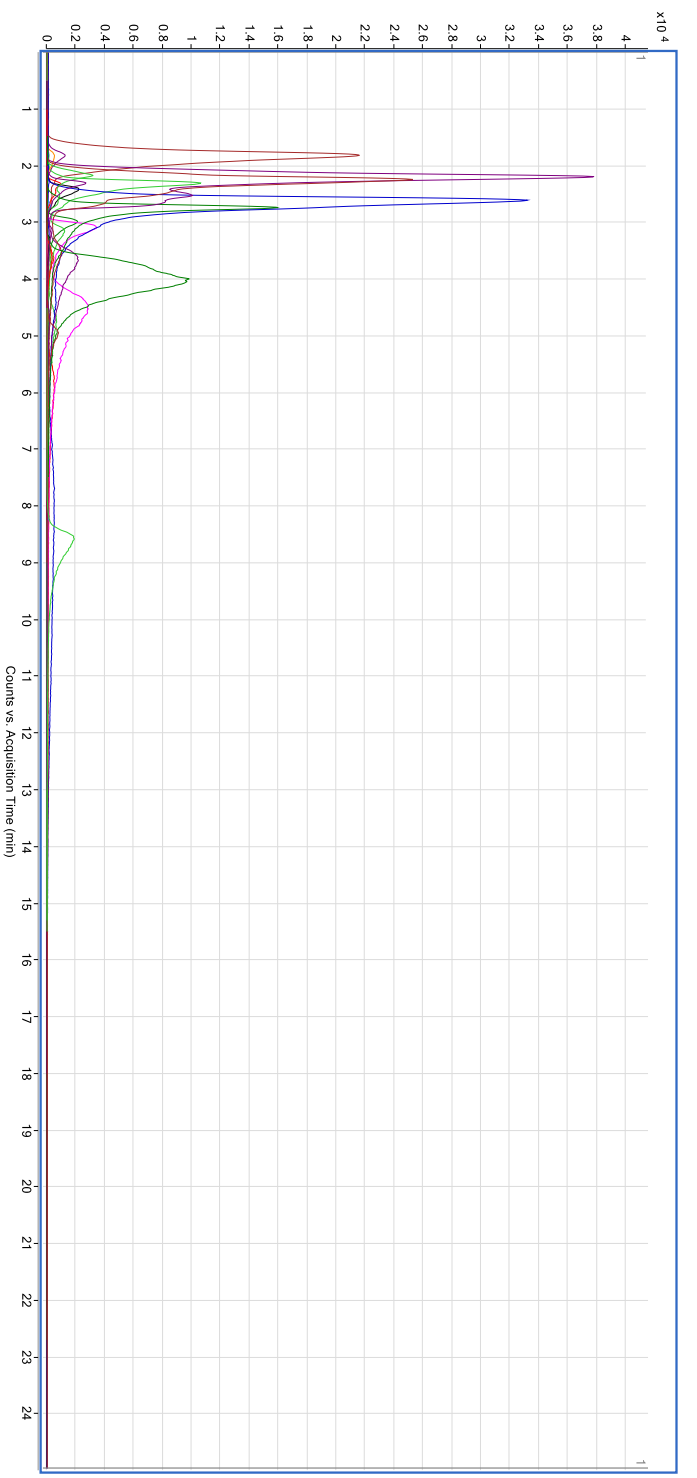
Supplementary material



Supplementary Fig. 1. SRM chromatograms from a multistandard solution and the chromatographic methods based on either the HILIC column (A), or the C_{18} column (B).



Supplementary Fig. 2. SRM chromatogram from sweat spiked with 50 ng/mL of each amino acid by using c-µSPE as sample preparation strategy.

Chapter VIII

Metabolomics analysis of human sweat collected after moderate exercise

Metabolomics analysis of human sweat collected after moderate exercise

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Metabolomics analysis of human sweat collected after moderate exercise

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Abstract

Sweat is a promising biofluid scarcely used in clinical analysis despite its no invasive sampling. A more frequent clinical use of sweat requires to know its whole composition, especially concerning to no polar compounds, and the development of analytical strategies for its characterization. The aim of the present study was to compare different sample preparation strategies to maximize the detection of metabolites in sweat from humans collected after practicing moderate exercise. Special emphasis was put on no polar compounds as they have received scant attention in previous studies dealing with this biofluid. Sample preparation by liquid–liquid extraction (LLE) using extractants with different polarity index was compared to deproteination. Then, derivatization by methoxymation with subsequent silylation was compared to direct analysis of sweat extracts to check the influence of derivatization on the subsequent determination of volatile organic compounds (VOCs). A number of 135 compounds were tentatively identified by combining spectral and retention time information after analysis by gas chromatography coupled to mass spectrometry in high resolution mode (GC–TOF/MS). Lipids, VOCs, benzenoids and other interesting metabolites such as alkaloids and ethanolamines were identified. Among the tested protocols, methyoxiamination plus silylation after LLE with dichloromethane was the best option to obtain a representative snapshot of sweat metabolome collected from different body parts after moderate exercise. Passive and active sweat pools from a cohort of volunteers (n=6) were compared to detect compositional differences which can be explained by the sampling process and sweating induction. As most of the identified compounds are metabolites involved in key biochemical pathways, this study opens new opportunities to extend the applicability of human sweat as a source of metabolite biomarkers of pathologies or specific processes such as dehydration or nutritional unbalance.

Keywords: metabolomics, active sweat, passive sweat, exercise, gas chromatography–mass spectrometry, sample preparation.

1. Introduction

Sweat is mainly composed by water (99%), electrolytes, small molecules (*e.g.,* amines, carboxylic acids, amino acids), complex biomolecules such as proteins and antimicrobial peptides, and xenobiotics such as drugs, cosmetics, and ethanol, among others [1]. Due to the aqueous nature of sweat, the majority of the compounds identified in this biofluid are polar, but also no polar or intermediate polarity compounds have been identified in this biofluid. For example, in 1974 Förström *et al.* detected prostaglandins in sweat [2]. Fatty acids have also been detected in sweat by GC–MS and by GC coupled to Fourier transform infrared spectroscopy (GC–FTIR) [3]. Thus, sweat analysis showed the presence of several C_6–C_{10} straight chains, branched, and unsaturated acids [3], but also conventional acids such as lauric, myristic, palmitic, oleic and stearic acids were detected [4]. Moreover, short-chain fatty acids have been determined in feet sweat after either Soxhlet extraction with diethyl ether [5] or headspace solid-phase microextraction [6] with subsequent GC–MS analysis in both cases.

Characterization studies of sweat by nuclear magnetic resonance (NMR) have revealed that the main constituents of human sweat are compounds involved in primary and secondary biological functions [7]. Polar compounds such as amino acids, amines, sugars, lactate, glycerol, ethanol, formate, acetaldehyde, urea, inositol and compounds involved in the citric acid cycle (*e.g.,* pyruvate, fumarate or aconitate) were mainly detected. However, NMR analysis only allowed detection of some characteristic groups of lipids. This fact could be explained by the low concentration of lipids in the collected samples (< 0.01 mM) and the low resolution of ^{1}H-NMR to discriminate among lipid families. In fact, only some signals corresponding to -CH_2- and -CH_3 groups were detected [7,8]. Other characterization study of sweat by LC–QTOF MS/MS confirmed that most of human sweat components are involved in key biochemical pathways [9]. Among them, the main families of detected metabolites were polar compounds such as amino acids, metabolites involved in purine nucleosides degradation, dicarboxylic acids, sugars, nutrients and exogenous compounds. On the contrary, only 4 no polar compounds (3 fatty acids and 1 sphingolipid) were detected [9]. A recent study on human sweat analysis by GC–TOF/MS in high resolution mode allowed the identification of 66 compounds, most of them also polar compounds (18 amino acids, 12 carboxylic acids, 12 sugars and 4 benzene derivatives), and only 14 no polar lipids were detected [10].

All these previous studies had in common the collection of the known as passive sweat —that collected from individuals at rest—, although two different sampling strategies were used. In two studies based on MS, passive sweat was collected by the commercial Macroduct device, which is clinically used for the diagnostic of some diseases such as cystic fibrosis [11]. In the study based on NMR analysis, sweat was collected by special tools designed by the authors, which included glass rollers with dull surfaces and holders for sample collection when the sweat quantity was limited, and special pipettes with reverse capillaries for sweat drop collection in the case of profuse sweat secretion [7].

The sampling devices employed could explain the differences in sweat composition, and the lack of no polar compounds [7]. Sweating is usually stimulated by heat or chemical induction (for instance, using pilocarpine), but also by exercise practice at different intensity levels [12]. The first two protocols, based on temperature increase or chemical stimulation, allow collection of passive sweat, while that collected after exercise practice provides the known as active sweat, which can set differences at compositional level as compared to passive sweat.

Recently, sensors to evaluate nutritional unbalance, dehydration and clinical biomarkers in sweat have been developed. As an example, a real-time colorimetric sensor that detects dehydratation, normal condition and over-hydratation within minutes has recently been designed for hydratation balance [13]. As the analysis is carried out in sweat, it is no invasive and makes possible real-time feedback [13]. Other example is a mechanically flexible and fully integrated sensor array for multiplexed *in situ* perspiration analysis that has allowed the simultaneous and selective screening of a panel of biomarkers in sweat. This sensor measures sweat metabolites (such as glucose and lactate) and electrolytes (such as sodium and potassium ions), as well as the skin temperature (to calibrate the sensors response). The portable system is used to evaluate the response of humans engaged in prolonged indoor and outdoor physical activities, and to make real-time assessment of the physiological state of the individuals. This platform enables a wide range of personalized diagnostic and physiological monitoring applications [14].

The aim of the present study was to optimize a method for untargeted metabolomics analysis of active sweat, with special emphasis on no polar compounds, considering that lipids have been scarcely detected in active sweat probably owing to both their low concentration and the sampling strategy. Active sweat will be compared to passive

sweat to evaluate compositional differences associated to the stimulated perspiration process.

2. Experimental

2.1. Reagents

Optima™ LC–MS grade acetonitrile from Thermo Fisher Scientific (Pittsburgh, PA, USA), LC–MS grade methanol, GC grade dichloromethane, and Trace SELECT® grade ethyl acetate and *n*-hexane from Sigma–Aldrich (St. Louis, MO, USA) were used for sample preparation. Deionized water (18 mΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used for standards preparation. Pyridine and methoxyamine hydrochloride from Sigma–Aldrich were used as solvent and reagent, respectively, for methoxymation. Bis-(trimethylsilyl) fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were used as silylation agents in the derivatization step. MS-grade perfluorotri-*n*-butylamine (PFTBA) from Agilent Technologies (Santa Clara, CA, USA) was used for daily mass calibration in GC–TOF/MS. A standard mixture containing ten linear alkanes from C10 to C40 designed for performance tests in GC from Sigma–Aldrich was used to establish the retention index (RI) calibration model. HPLC–grade *n*-hexane and acetone from Scharlab (Barcelona, Spain) were used for washing the injection syringe. Standards of D-galactose, D-glucose and myo-inositol from Sigma–Aldrich were used to confirm sugars identification.

2.2. Instruments and apparatus

A vortex shaker from IKA (Wilmington, NC, USA) was used to promote deproteination or liquid–liquid extraction (LLE). A Legend Micro 21R microcentrifuge from Thermo Fisher Scientific was used to centrifuge and separate the immiscible phases. A Concentrator plus™ from Eppendorf (Hamburg, Germany) with three application modes (aqueous, alcohol or high vapor pressure) was used to evaporate the extracts and the deproteinated sample before derivatization. A ThermoMixer C from Eppendorf was used to heat and mix solutions involved in the derivatization steps. An Agilent 7890A Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer

equipped with electron impact (EI) source was used for analysis. The analytical sample was thus monitored in high resolution mode. MassHunter GC–QTOF Acquisition software (version B.07, Agilent Technologies) was used to control data acquisition and set the parameters for optimum operation.

2.3. Sweat collection

Active sweat was sampled from three different body areas (forehead, chest and back) by using a micropipette (from 20 to 200 μL) from Kartell (Noviglio, Italy) after 30 min of moderate exercise. Previously, the skin area was cleaned with ethanol and then with distilled water. The use of deodorants, perfumes and cosmetics was excluded at least one day prior to sweat collection. The sweat was transferred to plastic microEppendorf tubes and stored at −80 ºC until analysis. A total sweat volume of at least 100 μL was collected per individual and body area.

Passive sweat was sampled by using the Macroduct® Sweat Analysis System (Wescor, Utah, USA) from donors at rest, as described by Delgado-Povedano *et al.* [10]. A total sweat volume of at least 70 μL was collected per individual from the forearm.

Sweat samples for development and characterization of the method were kindly donated by 6 healthy volunteers (4 men and 2 women) at Reina Sofía University Hospital (Córdoba, Spain). All subjects gave their informed consent for sweat sampling. No restrictions about diet or age were considered. All steps from sweat sampling to analysis were carried out in accordance with the ethical principles of the World Medical Association (Helsinki Declaration, 2004).

2.4. Sample treatment

Active and passive sweat pools were prepared by taking 70 μL of sweat from each participant. Then, different experimental strategies were tested with aliquots of both pools to select that providing maximum metabolite coverage:

(i) Protocol 1: Liquid–liquid extraction (LLE) with different extractants and direct analysis of the extracts. Three organic solvents (hexane, dichloromethane and ethyl acetate) were evaluated as extractants. In all cases, a 12.5 μL aliquot of sample was vortexed with 75 μL of extractant in a glass insert at room

temperature for 5 min and centrifuged for 5 min at 10000×*g*. To assure the absence of water in the extract to be injected in the GC–MS system, the vial was subjected to −20 ºC to freeze the aqueous fraction; then, the organic phase was transferred to a new glass insert for analysis.

(ii) Protocol 2: LLE and methoxymation+sylilation prior to analysis. After LLE followed by centrifugation as in protocol 1, the organic phase was isolated and located into a new glass insert for evaporation to dryness. Then, the solid residue was reconstituted with 10 μL of 40 mg/mL methoxyamine hydrochloride in pyridine. After adding the methoxymation agents, the mixture was shaken and maintained at 30 °C for 90 min. Then, 45 μL of 100:1 (v/v) BSTFA–TMCS mixture was added for silylation and the system was shaken for 30 s and maintained at 37 °C for 30 min prior to GC–MS analysis.

(iii) Protocol 3: Deproteination with methanol–acetonitrile before methoxymation+silylation prior to analysis. With this aim, a 12.5 μL aliquot was vortexed with 75 μL of 70:30 (v:v) methanol–acetonitrile in a glass insert at room temperature for 5 min, then centrifuged for 5 min at 10000×*g*. The liquid phase was isolated and located into a new glass insert, evaporated to dryness before methoxymation plus silylation and analysis as described in Protocol 2.

2.5. GC–TOF/MS analysis

GC–TOF/MS analyses were performed following the method proposed by Delgado-Povedano *et al.* [10] by using a fused silica DB-5MS-UI 30 m×0.25 mm i.d.×0.25 μm film thickness capillary column. All samples were analyzed in duplicate.

2.6. Identification of metabolites

Identification was carried out by searching MS spectra in theNIST11 and Fiehn databases [15] based on the criteria described by Delgado-Povedano *et al.* [10]. The match factor, reverse match factor and RI values in NIST database supported identifications. Fig. S1 shows the RI calibration graph and the equation fitting the model, both obtained as in Ref. [10]. Identification of sugars (D-galactose, D-glucose and myo-inositol) was confirmed by analysis of standards derivatized by the described protocol.

2.7. Data processing and statistical analysis

Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process all data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to treat all data files. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of molecular features (MFs) considered as potential compounds defined by the *m/z* value and retention time (RT) of one representative ion for each chromatographic peak. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions, the accuracy error at 50 ppm and the window size factor at 200 units. Finally, the MFs from data pretreatment were exported (.cef file) for recursive analysis. For this purpose, the Quantitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to reintegrate all potential compounds found in all analyzed samples. The resulting table was exported in comma separated value format (.csv file) into the Mass Profiler Professional software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

3. Results and discussion

3.1. Liquid–liquid extraction of active sweat with no polar solvents and influence of derivatization on the detection coverage

Sweat samples are not ready for direct analysis by GC–MS because of the aqueous nature of this excreted biofluid. Therefore, a sample preparation step is required to define suited protocols for the analysis and characterization of sweat composition. Deproteination with a methanol–acetonitrile mixture has previously been proposed as the best approach for untargeted analysis of low-molecular weight compounds in sweat sampled by a passive protocol [10]. However, no especial attention was paid to the scant no polar metabolites identified by MS.

The research proposed here is intended to characterize active sweat produced after a moderate exercise session. Special attention was paid to no polar compounds, but without losing the capability of detecting polar and mid-polar compounds. For this purpose, three different extractants covering a mid-polar/no polar range —ethyl acetate (polarity index PI

4.4), dichloromethane (PI 3.1) and hexane (PI 0.0)— were selected to evaluate their extraction performance. Sweat aliquots of the pool were 1:6 extracted with each solvent and the resulting extracts were subjected to the derivatization protocol, consisting of methoxymation and silylation steps, prior to GC–TOF/MS analysis. Fig. S2 shows the base peak chromatograms (BPCs) provided by analysis of the derivatized extracts obtained with the three extractants, which clearly revealed the dichloromethane extract as that providing a higher number of chromatographic peaks with the highest sensitivity. Fig. 1A shows the Venn diagram comparing the number of compounds tentatively identified in the derivatized extracts. Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts. As can be seen, the number of identified compounds was clearly higher in the dichloromethane extract, which agrees with the results obtained by Delgado-Povedano *et al.,* who compared dichloromethane and ethyl acetate for characterization of passive sweat [10]. The list of identified compounds in the three analytical samples is shown in Table 1. The analysis of sweat subjected to LLE with dichloromethane allowed the tentative identification of 121 compounds as compared to ethyl acetate and hexane with 87 and 43 compounds, respectively. One fact to be emphasized is that 98 and 86% of the compounds identified in the ethyl acetate and hexane extracts, respectively, were also detected in the dichloromethane extract.

(A)

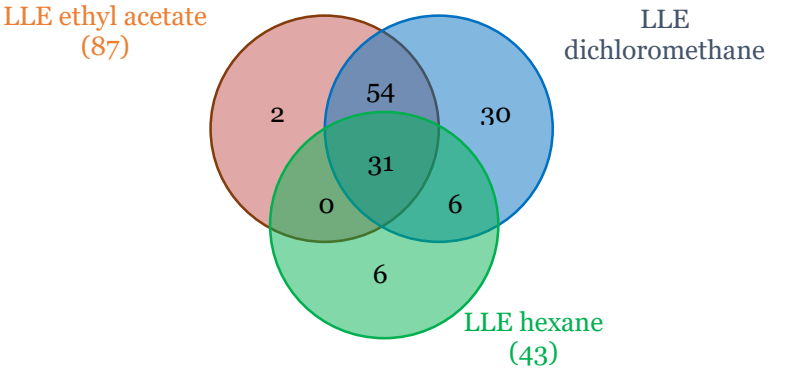

Fig. 1 (A) Venn diagram comparing the number of compounds tentatively identified in ethyl acetate, dichloromethane and hexane derivatized extracts of active sweat. (Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts).

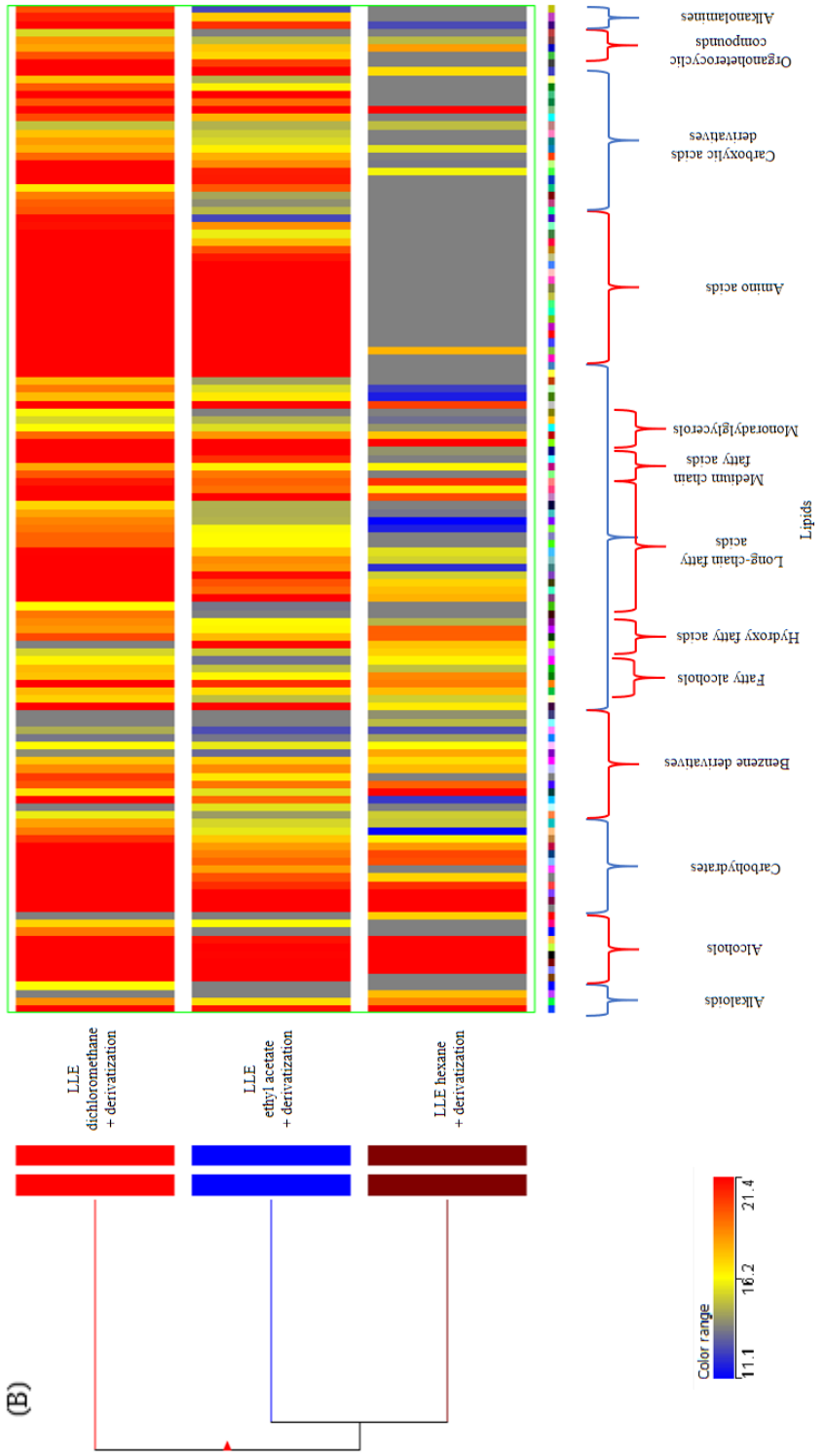

Fig. 1 (B) Heat map comparing relative concentrations of compounds tentatively identified in ethyl acetate, dichloromethane and hexane derivatized extracts of active sweat. Color coding indicates differences in normalized concentration for each metabolite.

Table 1. Compounds tentatively identified by GC–TOF/MS in ethyl acetate, dichloromethane and hexane extracts of active sweat after derivatization.

Compound[a]	RT (min)	Formula	Family	LLE + derivatization		
				Ethyl acetate	Dichloro-methane	Hexane
Nicotine	10.89	$C_{10}H_{14}N_2$	Alkaloids			✓
Cotinine	15.01	$C_{10}H_{12}N_2O$	Alkaloids	✓	✓	✓
Caffeine	16.52	$C_8H_{10}N_4O_2$	Alkaloids	✓	✓	✓
Ethanolamine	9.36	C_2H_7NO	Alkanolamines	✓	✓	
Diethanolamine	9.60	$C_4H_{11}NO_2$	Alkanolamines		✓	
	11.67				✓	
Triethanolamine	13.99	$C_6H_{15}NO_3$	Alkanolamines	✓	✓	
Valine	*6.75*	$C_5H_{11}NO_2$	Amino acids		✓	
	8.62			✓	✓	
Alanine	6.94	$C_3H_7NO_2$	Amino acids	✓	✓	
Glycine	*7.24*	$C_2H_5NO_2$	Amino acids	✓	✓	
	9.92			✓	✓	
Leucine	7.77	$C_6H_{13}NO_2$	Amino acids		✓	
Isoleucine	*8.10*	$C_6H_{13}NO_2$	Amino acids	✓	✓	
	9.73			✓	✓	
Serine	*9.24*	$C_3H_7NO_3$	Amino acids	✓	✓	
	10.64			✓	✓	
Proline	9.81	$C_5H_9NO_2$	Amino acids	✓	✓	
Threonine	10.98	$C_4H_9NO_3$	Amino acids	✓	✓	
Sarcosine	11.19	$C_3H_7NO_2$	Amino acids		✓	
Aspartic acid	*11.51*	$C_4H_7NO_4$	Amino acids	✓		
	12.71		Amino acids	✓	✓	

Continuation Table 1

Aminomalonic acid	12.08	$C_3H_5NO_4$	Amino acids	✓	✓	
Pyroglutamic acid	*12.48*	$C_5H_7NO_3$	Amino acids	✓	✓	
	12.74		Amino acids	✓	✓	
γ-Aminobutyric acid	12.85	$C_4H_9NO_2$	Amino acids	✓	✓	
α-Aminoadipic acid	13.05	$C_6H_{11}NO_4$	Amino acids		✓	
2-Aminoheptanedioic acid	13.70	$C_7H_{13}NO_4$	Amino acids		✓	
Ornithine	*13.86*	$C_5H_{12}N_2O_2$	Amino acids	✓	✓	
	16.10			✓	✓	
Glutamic acid	13.91	$C_5H_9NO_4$	Amino acids		✓	
Phenylalanine	14.01	$C_9H_{11}NO_2$	Amino acids	✓	✓	
Asparagine	14.49	$C_4H_8N_2O_3$	Amino acids	✓	✓	
Lysine	*14.92*	$C_6H_{14}N_2O_2$	Amino acids	✓	✓	
	17.19			✓	✓	
Glutamine	15.63	$C_5H_{10}N_2O_3$	Amino acids	✓	✓	
Citrulline	*16.18*	$C_6H_{13}N_3O_3$	Amino acids		✓	
	18.97				✓	
Histidine	17.16	$C_6H_9N_3O_2$	Amino acids	✓	✓	
Tyrosine	17.36	$C_9H_{11}NO_3$	Amino acids		✓	
Tryptophan	19.57	$C_{11}H_{12}N_2O_2$	Amino acids		✓	
	19.78				✓	
	19.89				✓	
	19.96				✓	
Indane	6.05	C_9H_{10}	Benzene derivatives	✓	✓	✓
Benzoic acid	9.14	$C_7H_6O_2$	Benzene derivatives (acids)	✓	✓	✓
4-Acetyloxybenzoic acid	14.50	$C_9H_8O_4$	Benzene derivatives (acids)	✓	✓	✓
4-Hydroxyphenyllactic acid	16.95	$C_9H_{10}O_4$	Benzene derivatives (acids)	✓	✓	
α-Keto-4-hydroxyben-zenepropanoic acid	17.04	$C_9H_8O_4$	Benzene derivatives (acids)		✓	

Continuation Table 1

β-Ethenylbenzeneethanol	6.05	$C_{10}H_{12}O$	Benzene derivatives (alcohols)	✓	✓	✓
Cathecol	10.08	$C_6H_6O_2$	Benzene derivatives (alcohols)			✓
3-Methylcathecol	11.06	$C_7H_8O_2$	Benzene derivatives (alcohols)			✓
2,4-Ditertbutylphenol	12.63	$C_{14}H_{22}O$	Benzene derivatives (alcohols)	✓	✓	✓
Pyrogallol	13.70	$C_6H_6O_3$	Benzene derivatives (alcohols)			✓
Triclosan	19.14	$C_{12}H_7Cl_3O_2$	Benzene derivatives (clorinated)			✓
Carbamate	8.40	CH_3NO_2	Carbamic acids	✓	✓	
Trehalose	24.09	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)		✓	
Erythrulose	14.56	$C_4H_8O_4$	Carbohydrates (monosaccharides)		✓	
Sorbitol	16.48	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)		✓	
Galactose	*16.83*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)		✓	✓
	17.06				✓	✓
Glucose	*16.90*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)		✓	✓
	17.10				✓	✓
Glyceric acid	10.22	$C_3H_6O_4$	Carbohydrates (sugar acids)	✓	✓	✓
Threonic acid	12.96	$C_4H_8O_5$	Carbohydrates (sugar acids)	✓	✓	
Gluconic acid	17.92	$C_6H_{12}O_7$	Carbohydrates (sugar acids)	✓	✓	
Glycerol	9.43	$C_3H_8O_3$	Carbohydrates (sugar alcohols)	✓	✓	✓
Erythritol	12.53	$C_4H_{10}O_4$	Carbohydrates (sugar alcohols)	✓	✓	✓
Mannitol	17.29	$C_6H_{14}O_6$	Carbohydrates (sugar alcohols)		✓	
Inositol	*18.19*	$C_6H_{12}O_6$	Carbohydrates (sugar alcohols)		✓	✓
myo-Inositol	18.80				✓	✓
2,3-Butanediol	5.82	$C_4H_{10}O_2$	Other alcohols and polyols		✓	✓
Propylene glycol	5.98	$C_3H_8O_2$	Other alcohols and polyols	✓	✓	✓
1,3-Butanediol	6.61	$C_4H_{10}O_2$	Other alcohols and polyols	✓	✓	✓

Continuation Table 1

Pinacol	7.75	$C_6H_{14}O_2$	Other alcohols and polyols	✓	✓	✓
1,2-Butanediol	8.05	$C_4H_{10}O_2$	Other alcohols and polyols		✓	
Diethylene glycol	9.04	$C_4H_{10}O_3$	Other alcohols and polyols	✓	✓	
1-Methylene-3-methylbutane-1,3-diol	9.19	$C_6H_{12}O_2$	Other alcohols and polyols	✓	✓	
2-(2-Butoxyethoxy)ethanol	10.37	$C_8H_{18}O_3$	Other alcohols and polyols	✓	✓	
1,1,1-Tris(hydroxy-methyl)propane	11.06	$C_6H_{14}O_3$	Other alcohols and polyols			✓
Succinic acid	10.04	$C_4H_6O_4$	Dicarboxylic acids	✓	✓	
Itaconic acid	10.41	$C_5H_6O_4$	Dicarboxylic acids	✓	✓	✓
Citraconic acid	10.50	$C_5H_6O_4$	Dicarboxylic acids	✓	✓	✓
Fumaric acid	10.54	$C_4H_4O_4$	Dicarboxylic acids	✓	✓	
Glutaric acid	11.27	$C_5H_8O_4$	Dicarboxylic acids	✓	✓	
Malic acid	12.30	$C_4H_6O_5$	Dicarboxylic acids	✓	✓	
Adipic acid	12.56	$C_6H_{10}O_4$	Dicarboxylic acids	✓	✓	
Azelaic acid	15.91	$C_9H_{16}O_4$	Dicarboxylic acids	✓	✓	
Aconitic acid	15.35	$C_6H_6O_6$	Tricarboxylic acids	✓	✓	
Pyruvic acid	6.10	$C_3H_4O_3$	Carboxylic acids (keto acids)	✓	✓	
α-Ketoglutaric acid	13.38	$C_5H_6O_5$	Carboxylic acids (keto acids)	✓	✓	
Lactic acid	*5.79*	$C_3H_6O_3$	Carboxylic acids (hydroxy acids)			✓
	6.25			✓	✓	✓
Glycolic acid	6.51	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)	✓	✓	
Caproic acid	6.51	$C_6H_{12}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	
Caprylic acid	9.36	$C_8H_{16}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	✓
Pelargonic acid	10.69	$C_9H_{18}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	✓
Capric acid	11.97	$C_{10}H_{20}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	✓

Continuation Table 1

Lauric acid	14.33	$C_{12}H_{24}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	
Tridecylic acid	15.43	$C_{13}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Mirystoleic acid	16.25	$C_{14}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Myristic acid	16.48	$C_{14}H_{28}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Pentadecylic acid	17.48	$C_{15}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Palmitic acid	18.44	$C_{16}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Palmitoleic acid	18.19	$C_{16}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
10-Heptadecenoic acid	19.12	$C_{17}H_{32}O$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Margaric acid	19.36	$C_{17}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Arachidonic acid	19.83	$C_{20}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Linoleic acid	19.83	$C_{18}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Oleic acid	20.01	$C_{18}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓	
Stearic acid	20.24	$C_{18}H_{36}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Arachidic acid	21.90	$C_{20}H_{40}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	
Lignoceric acid	24.88	$C_{24}H_{48}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)		✓	

Continuation Table 1

Hexacosanoic acid	26.41	$C_{26}H_{52}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)	✓	✓	
Dipropylacetic acid	9.36	$C_8H_{16}O_2$	Lipids (methyl-branched fatty acids)	✓	✓	
2-Hydroxybutyric acid	7.25	$C_4H_8O_3$	Lipids (hydroxy fatty acids)		✓	
3-Hydroxybutyric acid	7.81	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓	✓
3-Hydroxyisovaleric acid	7.85	$C_5H_{10}O_3$	Lipids (hydroxy fatty acids)		✓	✓
2-Hydroxyisocaproic acid	8.91	$C_6H_{12}O_3$	Lipids (hydroxy fatty acids)	✓		
5-Hydroxy-n-valeric acid	10.29	$C_5H_{10}O$	Lipids (hydroxy fatty acids)	✓	✓	
Erucylamide	24.51	$C_{22}H_{43}NO$	Lipids (fatty amides)	✓	✓	✓
Mono(2-ethylhexyl)adipate	21.50	$C_{14}H_{26}O_4$	Lipids (fatty acid esters)	✓	✓	✓
1-Monoisobutyroylglycerol	12.08	$C_7H_{14}O_4$	Lipids (acylglycerides)	✓	✓	
2-Monopalmitoylglycerol	22.76	$C_{19}H_{38}O_4$	Lipids (acylglycerides)		✓	
1-Monopalmitoylglycerol	23.02	$C_{19}H_{38}O_4$	Lipids (acylglycerides)	✓	✓	✓
2-Monostearoylglycerol	24.20	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓	✓
1-Monostearoylglycerol	24.45	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓	✓
1-Monoarachidoylglycerol	25.86	$C_{23}H_{46}O_4$	Lipids (acylglycerides)	✓	✓	✓
Dehydroabietic acid	21.54	$C_{20}H_{28}O_2$	Lipids (prenol lipids-diterpenes)	✓	✓	
Squalene	24.76	$C_{30}H_{50}$	Lipids (prenol lipids-triterpenes)		✓	
Hedione	14.35	$C_{13}H_{22}O_3$	Lipids (lineolic acids and derivatives-jasmonic acids)	✓	✓	
Cholesta-3,5-diene	25.47	$C_{27}H_{44}$	Lipids (steroids and steroids derivatives)	✓	✓	✓
1-Dodecanol	13.35	$C_{12}H_{26}O$	Lipids (fatty alcohols)	✓	✓	✓
1-Tetradecanol	15.58	$C_{14}H_{30}O$	Lipids (fatty alcohols)	✓	✓	✓
1-Hexadecanol	17.62	$C_{16}H_{34}O$	Lipids (fatty alcohols)		✓	✓
1-Octadecanol	19.47	$C_{18}H_{38}O$	Lipids (fatty alcohols)		✓	
Phosphate	9.42	PO_4^{3-}	No metal oxoanionic compounds	✓	✓	

Continuation Table 1

2-Furoic acid	7.46	$C_5H_4O_3$	Organoheterocyclic compounds	✓	✓	✓
Uracil	10.34	$C_4H_4N_2O_2$	Organoheterocyclic compounds		✓	
Uridine	15.12	$C_9H_{12}N_2O_6$	Organoheterocyclic compounds		✓	
Urocanic acid	15.63	$C_6H_6N_2O_2$	Organoheterocyclic compounds	✓	✓	
Xanthine	18.13	$C_5H_4N_4O_2$	Organoheterocyclic compounds		✓	
Uric acid	18.89	$C_5H_4N_4O_3$	Organoheterocyclic compounds	✓	✓	
Urea	*8.10*	CH_4N_2O	Ureas	✓	✓	
	9.00			✓	✓	
Borate	9.64	BO_3^{3-}	Exogenous	✓		
Methylparaben	12.46	$C_8H_8O_3$	Exogenous	✓	✓	✓

[a] Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts.

On the other hand, the hexane extract allowed identifying less than 31% of the sweat components found in the dichloromethane extract, while the ethyl acetate extract covered approximately 70% of them. Dichloromethane and ethyl acetate provided very close compositions, but the former allowed detecting no polar compounds with higher sensitivity. This is clear in the heat map shown in Fig. 1B that compares the three extractants. As can be seen, only few compounds were quantitatively extracted with hexane or ethyl acetate as compared to dichloromethane. Attending to these results, dichloromethane would be recommended as solvent to extract no polar and also polar compounds from sweat.

The inclusion of a generic derivatization procedure such as methoxymation followed by silylation enables to enhance the detection of no volatile components. On the other hand, VOCs could be detected by direct GC–MS analysis. This approach was used by Zeng *et al.* in 1991 for the first time for the identification of VOCs in human sweat [3]. At present, it is well known that sweat may contain both a wide variety of no volatile compounds but also VOCs. With these premises, all sweat extracts (ethyl acetate, dichloromethane and hexane extracts) were also directly analyzed without derivatization. Fig. S3 shows the BPCs provided by direct analysis of the three extracts. As can be seen, the

chromatographic profiles provided by the derivatized extracts clearly reported more peaks than the underivatized extracts, but also the sensitivity was enhanced when derivatization was implemented. This result was especially remarkable for the dichloromethane extract as compared to ethyl acetate and hexane extracts.

Fig. 2 compares the number of identified compounds in the dichloromethane extract with and without derivatization. Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts. Direct analysis only made possible to identify 14 compounds, 7 of them (isobutylparaben, propylparaben, triclosan, phenylethyl alcohol, α,α-dimethylbenzene-methanol, erucylamide and 1-undecyne) exclusively identified by direct analysis of the extract.

The same identification results were obtained for ethyl acetate and hexane samples since derivatization of the extract clearly led to higher identification rates than direct analysis. Therefore, methoxymation plus sylilation after LLE with dichloromethane seems to be the suited sample preparation approach for sweat analysis by GC–TOF/MS.

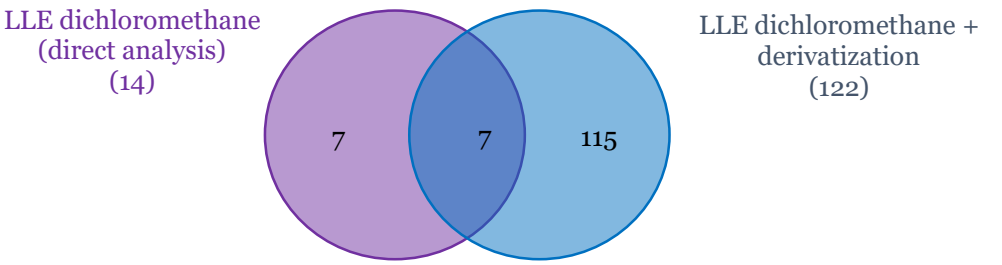

Fig. 2. Venn diagram comparing the number of compounds tentatively identified in dichloromethane extracts of active sweat after derivatization and direct analysis. (Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts).

3.2. Comparison of liquid–liquid extraction and deproteination for untargeted analysis of active sweat

After knowing the chemical diversity of compounds found in sweat after LLE, the performance of a deproteination protocol using methanol–acetonitrile was tested. Sweat

aliquots were 1:6 mixed with a 70:30 (v/v) methanol–acetonitrile mixture. The same derivatization protocol was used to enhance identification as previously assayed. Fig. 3 shows the Venn diagram comparing the number of compounds tentatively identified in dichloromethane extract and deproteinated sweat.

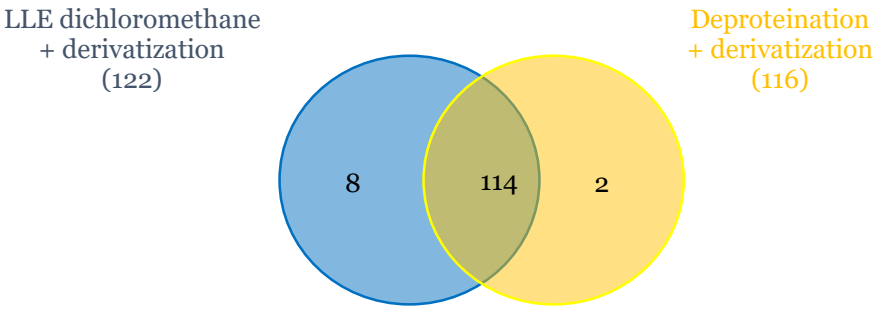

Fig. 3. Venn diagram comparing the number of compounds tentatively identified in dichloromethane extract and in active sweat deproteinated with methanol–acetonitrile. Derivatization was applied to both extracts. (Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts).

The criterion used to validate identifications was adopted here. The list of the compounds identified in these two analytical samples is shown in Table 2. The analysis of sweat subjected to deproteination reported 116 compounds, a number very close to that obtained after LLE. One fact to be emphasized is that 98% of the compounds detected by deproteination of sweat were also detected in the dichloromethane extracts. Three of the compounds detected only in dichloromethane extracts fit three main unsaturated fatty acids: C18:1 ω-9, C18:2 ω-6 and C20:4 ω-6.

Concerning the sensitivity of the approach, only 32% of the compounds commonly detected in the analytical samples obtained by both sample preparation protocols reported a lower sensitivity in the dichloromethane extract as compared to the deproteined sample. Most of these compounds were amino acids, mainly essential amino acids such as valine, lysine, histidine and tryptophan. Fig. 4 shows the heat map that compares the relative concentrations of compounds detected using the two protocols. As can be seen, methoxymation plus silylation after LLE with dichloromethane seems to be the suited

sample preparation approach for analysis by GC–TOF/MS of active sweat after moderate exercise.

Table 2. Compounds tentatively identified by GC–TOF/MS in dichloromethane extract of active sweat and deproteinated sweat, after derivatization in both cases.

Compound[a]	**RT (min)**	**Formula**	**Family**	**Deproteination + derivatization**	**LLE dichloro-methane + derivatization**
Cotinine	15.01	$C_{10}H_{12}N_2O$	Alkaloids	✓	✓
Caffeine	16.52	$C_8H_{10}N_4O_2$	Alkaloids	✓	✓
Ethanolamine	9.36	C_2H_7NO	Alkanolamines	✓	✓
Diethanolamine	9.60	$C_4H_{11}NO_2$	Alkanolamines		✓
	11.67				✓
Triethanolamine	13.99	$C_6H_{15}NO_3$	Alkanolamines	✓	✓
Valine	*6.75*	$C_5H_{11}NO_2$	Amino acids	✓	✓
	8.62			✓	✓
Alanine	6.94	$C_3H_7NO_2$	Amino acids	✓	✓
Glycine	*7.24*	$C_2H_5NO_2$	Amino acids	✓	✓
	9.92			✓	✓
Leucine	7.77	$C_6H_{13}NO_2$	Amino acids		✓
Isoleucine	*8.10*	$C_6H_{13}NO_2$	Amino acids	✓	✓
	9.73			✓	✓
Serine	*9.24*	$C_3H_7NO_3$	Amino acids	✓	✓
	10.64			✓	✓
Proline	9.81	$C_5H_9NO_2$	Amino acids	✓	✓
Threonine	10.98	$C_4H_9NO_3$	Amino acids	✓	✓
Sarcosine	11.19	$C_3H_7NO_2$	Amino acids	✓	✓
Aspartic acid	12.71	$C_4H_7NO_4$	Amino acids	✓	✓
Aminomalonic acid	12.08	$C_3H_5NO_4$	Amino acids	✓	✓

Continuation Table 2

Pyroglutamic acid	*12.48*	$C_5H_7NO_3$	Amino acids	✓	✓
	12.74		Amino acids	✓	✓
γ-Aminobutyric acid	12.85	$C_4H_9NO_2$	Amino acids	✓	✓
α-Aminoadipic acid	13.05	$C_6H_{11}NO_4$	Amino acids	✓	✓
2-Aminoheptanedioic acid	13.70	$C_7H_{13}NO_4$	Amino acids	✓	✓
Ornithine	*13.86*	$C_5H_{12}N_2O_2$	Amino acids	✓	✓
	16.10			✓	✓
Glutamic acid	13.91	$C_5H_9NO_4$	Amino acids	✓	✓
Phenylalanine	14.01	$C_9H_{11}NO_2$	Amino acids	✓	✓
Asparagine	14.49	$C_4H_8N_2O_3$	Amino acids	✓	✓
Lysine	*14.92*	$C_6H_{14}N_2O_2$	Amino acids	✓	✓
	17.19			✓	✓
Glutamine	15.63	$C_5H_{10}N_2O_3$	Amino acids	✓	✓
Citrulline	*16.18*	$C_6H_{13}N_3O_3$	Amino acids	✓	✓
	18.97			✓	✓
Histidine	17.16	$C_6H_9N_3O_2$	Amino acids	✓	✓
Tyrosine	17.36	$C_9H_{11}NO_3$	Amino acids	✓	✓
Tryptophan	19.57	$C_{11}H_{12}N_2O_2$	Amino acids	✓	✓
	19.78			✓	✓
	19.89			✓	✓
	19.96			✓	✓
Indane	6.05	C_9H_{10}	Benzene derivatives	✓	✓
Benzoic acid	9.14	$C_7H_6O_2$	Benzene derivatives (acids)	✓	✓
4-Acetyloxybenzoic acid	14.50	$C_9H_8O_4$	Benzene derivatives (acids)	✓	✓
4-Hydroxyphenyllactic acid	16.95	$C_9H_{10}O_4$	Benzene derivatives (acids)	✓	✓
α-Keto-4-hydroxybenzenepropanoic acid	17.04	$C_9H_8O_4$	Benzene derivatives (acids)	✓	✓

Continuation Table 2

β-Ethenylben-zeneethanol	6.05	$C_{10}H_{12}O$	Benzene derivatives (alcohols)	✓	✓
Cathecol	10.08	$C_6H_6O_2$	Benzene derivatives (alcohols)	✓	✓
2,4-Ditertbutylphenol	12.63	$C_{14}H_{22}O$	Benzene derivatives (alcohols)	✓	✓
Pyrogallol	13.70	$C_6H_6O_3$	Benzene derivatives (alcohols)	✓	✓
Carbamate	8.40	CH_3NO_2	Carbamic acids	✓	
Trehalose	24.09	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)	✓	✓
Erythrulose	14.56	$C_4H_8O_4$	Carbohydrates (monosaccharides)	✓	✓
Sorbitol	16.48	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)	✓	✓
Galactose	*16.83*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓
	17.06			✓	✓
Glucose	*16.90*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓
	17.10			✓	✓
Glyceric acid	10.22	$C_3H_6O_4$	Carbohydrates (sugar acids)	✓	✓
Threonic acid	12.96	$C_4H_8O_5$	Carbohydrates (sugar acids)	✓	✓
Gluconic acid	17.92	$C_6H_{12}O_7$	Carbohydrates (sugar acids)	✓	✓
Glycerol	9.43	$C_3H_8O_3$	Carbohydrates (sugar alcohols)	✓	✓
Erythritol	12.53	$C_4H_{10}O_4$	Carbohydrates (sugar alcohols)	✓	✓
Mannitol	17.29	$C_6H_{14}O_6$	Carbohydrates (sugar alcohols)	✓	✓
Inositol	*18.19*	$C_6H_{12}O_6$	Carbohydrates (sugar alcohols)	✓	✓
myo-Inositol	18.80			✓	✓
2,3-Butanediol	5.82	$C_4H_{10}O_2$	Other alcohols and polyols	✓	✓
Propylene glycol	5.98	$C_3H_8O_2$	Other alcohols and polyols	✓	✓
1,3-Butanediol	6.61	$C_4H_{10}O_2$	Other alcohols and polyols	✓	✓
Pinacol	7.75	$C_6H_{14}O_2$	Other alcohols and polyols	✓	✓
1,2-Butanediol	8.05	$C_4H_{10}O_2$	Other alcohols and polyols		✓
Diethylene glycol	9.04	$C_4H_{10}O_3$	Other alcohols and polyols	✓	✓

Continuation Table 2

1-Methylene-3-methylbutane-1,3-diol	9.19	$C_6H_{12}O_2$	Other alcohols and polyols	✓	✓
2-(2-Butoxy-ethoxy)ethanol	10.37	$C_8H_{18}O_3$	Other alcohols and polyols	✓	✓
Succinic acid	10.04	$C_4H_6O_4$	Dicarboxylic acids	✓	✓
Itaconic acid	10.41	$C_5H_6O_4$	Dicarboxylic acids	✓	✓
Citraconic acid	10.50	$C_5H_6O_4$	Dicarboxylic acids	✓	✓
Fumaric acid	10.54	$C_4H_4O_4$	Dicarboxylic acids	✓	✓
Glutaric acid	11.27	$C_5H_8O_4$	Dicarboxylic acids	✓	✓
Malic acid	12.30	$C_4H_6O_5$	Dicarboxylic acids	✓	✓
Adipic acid	12.56	$C_6H_{10}O_4$	Dicarboxylic acids	✓	✓
Azelaic acid	15.91	$C_9H_{16}O_4$	Dicarboxylic acids	✓	✓
Aconitic acid	15.35	$C_6H_6O_6$	Tricarboxylic acids	✓	✓
Pyruvic acid	6.10	$C_3H_4O_3$	Carboxylic acids (keto acids)	✓	✓
α-Ketoglutaric acid	13.38	$C_5H_6O_5$	Carboxylic acids (keto acids)	✓	✓
Lactic acid	6.25	$C_3H_6O_3$	Carboxylic acids (hydroxy acids)	✓	✓
Glycolic acid	6.51	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)	✓	✓
Caproic acid	6.51	$C_6H_{12}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓
Caprylic acid	9.36	$C_8H_{16}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓
Pelargonic acid	10.69	$C_9H_{18}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓
Capric acid	11.97	$C_{10}H_{20}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓
Lauric acid	14.33	$C_{12}H_{24}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓
Tridecylic acid	15.43	$C_{13}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Mirystoleic acid	16.25	$C_{14}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓

Continuation Table 2

Myristic acid	16.48	$C_{14}H_{28}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Pentadecylic acid	17.48	$C_{15}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Palmitic acid	18.44	$C_{16}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Palmitoleic acid	18.19	$C_{16}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
10-Heptadecenoic acid	19.12	$C_{17}H_{32}O$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Margaric acid	19.36	$C_{17}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Arachidonic acid	19.83	$C_{20}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓
Linoleic acid	19.83	$C_{18}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓
Oleic acid	20.01	$C_{18}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)		✓
Stearic acid	20.24	$C_{18}H_{36}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Arachidic acid	21.90	$C_{20}H_{40}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓
Lignoceric acid	24.88	$C_{24}H_{48}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)	✓	✓
Hexacosanoic acid	26.41	$C_{26}H_{52}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)	✓	✓
Dipropylacetic acid	9.36	$C_8H_{16}O_2$	Lipids (methyl-branched fatty acids)	✓	✓
2-Hydroxybutyric acid	7.25	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓
3-Hydroxybutyric acid	7.81	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓

Continuation Table 2

3-Hydroxyisovaleric acid	7.85	$C_5H_{10}O_3$	Lipids (hydroxy fatty acids)	✓	✓
5-Hydroxy-n-valeric acid	10.29	$C_5H_{10}O_3$	Lipids (hydroxy fatty acids)	✓	✓
Erucylamide	24.51	$C_{22}H_{43}NO$	Lipids (fatty amides)	✓	✓
Mono(2-ethylhexyl)-adipate	21.50	$C_{14}H_{26}O_4$	Lipids (fatty acid esters)	✓	✓
1-Monoisobutyroyl-glycerol	12.08	$C_7H_{14}O_4$	Lipids (acylglycerides)	✓	✓
2-Monopalmitoylglycerol	22.76	$C_{19}H_{38}O_4$	Lipids (acylglycerides)	✓	✓
1-Monopalmitoylglycerol	23.02	$C_{19}H_{38}O_4$	Lipids (acylglycerides)	✓	✓
2-Monostearoylglycerol	24.20	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓
1-Monostearoylglycerol	24.45	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓
1-Monoarachidoyl-glycerol	25.86	$C_{23}H_{46}O_4$	Lipids (acylglycerides)	✓	✓
Dehydroabietic acid	21.54	$C_{20}H_{28}O_2$	Lipids (prenol lipids-diterpenes)	✓	✓
Squalene	24.76	$C_{30}H_{50}$	Lipids (prenol lipids-triterpenes)	✓	✓
Hedione	14.35	$C_{13}H_{22}O_3$	Lipids (lineolic acids and derivatives-jasmonic acids)	✓	✓
Cholesta-3,5-diene	25.47	$C_{27}H_{44}$	Lipids (steroids and steroids derivatives)	✓	✓
1-Dodecanol	13.35	$C_{12}H_{26}O$	Lipids (fatty alcohols)	✓	✓
1-Tetradecanol	15.58	$C_{14}H_{30}O$	Lipids (fatty alcohols)	✓	✓
1-Hexadecanol	17.62	$C_{16}H_{34}O$	Lipids (fatty alcohols)		✓
1-Octadecanol	19.47	$C_{18}H_{38}O$	Lipids (fatty alcohols)	✓	✓
Phosphate	9.42	PO_4^{3-}	No metal oxoanionic compounds	✓	✓
2-Furoic acid	7.46	$C_5H_4O_3$	Organoheterocyclic compounds	✓	✓
Uracil	10.34	$C_4H_4N_2O_2$	Organoheterocyclic compounds	✓	✓
Uridine	15.12	$C_9H_{12}N_2O_6$	Organoheterocyclic compounds	✓	✓
Urocanic acid	15.63	$C_6H_6N_2O_2$	Organoheterocyclic compounds	✓	✓
Xanthine	18.13	$C_5H_4N_4O_2$	Organoheterocyclic compounds		✓
Uric acid	18.89	$C_5H_4N_4O_3$	Organoheterocyclic compounds	✓	✓

Continuation Table 2

Urea	*8.10*	CH_4N_2O	Ureas	✓	✓
	9.00			✓	✓
Borate	9.64	BO_3^{3-}	Exogenous	✓	
Methylparaben	12.46	$C_8H_8O_3$	Exogenous	✓	✓

[a] Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the two analytical samples.

3.3. Identification of compounds in active sweat

Tentatively identified compounds covered a wide diversity of chemical families such as lipids, benzene derivatives, amino acids, carboxylic acids, organoheterocyclic compounds, alkaloids and carbohydrates, among others. This wide variety of compounds, particularly no polar compounds, would increase the interest of sweat for clinical studies, particularly for identification of biomarkers. Table 3 lists the identified compounds classified per chemical family. Identification parameters are detailed for each compound. As can be seen, lipids constitute the most abundant group of identified compounds; very different from previous characterization studies dealing with sweat [7,9]. The detection of lipids as a major metabolite class in human sweat samples confirms previous reports detailing the occurrence of lipids in eccrine sweat despite the aqueous nature of this sample [4]. Futhermore, the epidermis is a very active site of lipids synthesis. In the present study 20 fatty acids and conjugates, 8 fatty acid derivatives, 6 acylglycerides, 5 fatty alcohols, 2 prenol lipids, 1 steroid and one jasmonic acid derivative were identified in sweat. Fatty acids are secreted by apocrine glands and are required for the normal barrier function. Furthermore, the concentration of some fatty acids, especially C2-C11 fatty acids, have been correlated to microbial biotransformation [16]. Fatty acids detected in sweat were mostly saturated, ranging from short- to very long-chain fatty acids: caproic acid (C6:0), caprylic acid, (C8:0), pelargonic acid (C9:0), capric acid (C10:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), lignoceric acid (C24:0) and hexacosanoic acid (C26:0).

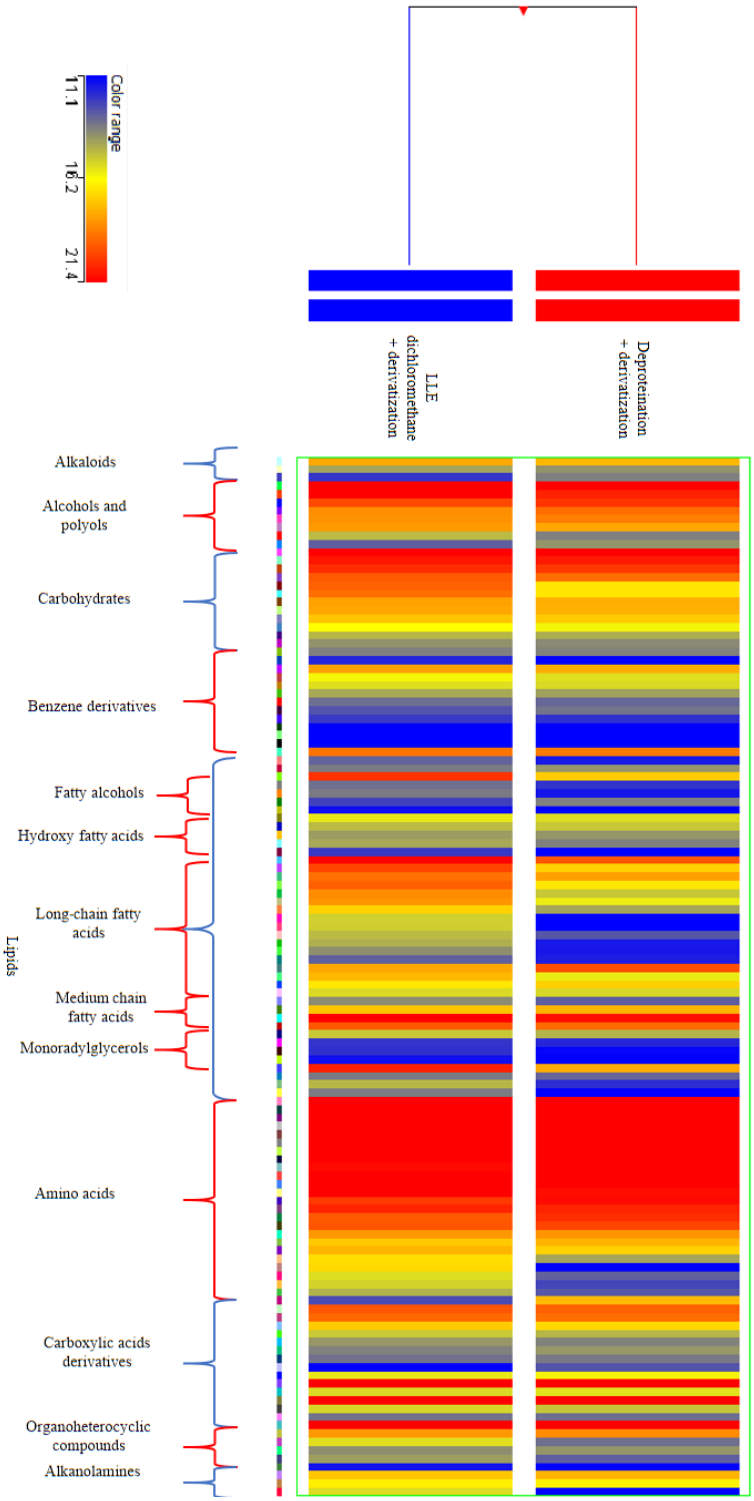

Fig. 4. Heat map comparing relative concentrations of compounds detected in the dichloromethane extract and in active sweat deproteinated with methanol–acetonitrile. Color coding indicates differences in normalized concentration for each metabolite.

Monounsaturated fatty acids (MUFAs) such as myristoleic acid (C14:1 ω-5), palmitoleic acid (C16:1 ω-7), 10-heptadecenoic acid (C17:1 ω-7) and oleic acid (C18:1 ω-9), and polyunsaturated fatty acids (PUFAs) such as linoleic acid (C18:2 ω-6) and arachidonic acid (C20:4 ω-6) were identified in sweat. Among them, C18:0 and C20:0, together with C18:1 ω-9 and C18:2 ω-6 constituted around 75% of all free fatty acids in sweat, in agreement with the results provided by Korea *et al.* [17].

Fatty acid derivatives detected in active sweat were mainly hydroxy fatty acids, which are present in human dermis and epidermis [18]: 2-hydroxy fatty acids (2-hydroxybutyric and 2-hydroxyisocaproic acids), 3-hydroxy fatty acids (3-hydroxybutyric and 3-hydroxyisovaleric acids) and a 5-hydroxy fatty acid (5-hydroxy-*n*-valeric acid). Other identified fatty acid derivatives were a fatty acid ester —mono(2-ethylhexyl)adipate—, a methyl-branched fatty acid (dipropylacetic acid) and a jasmonic acid derivative (hedione), which had not been previously detected in sweat. Only one fatty amide, erucylamide, was identified in sweat sampled after moderate active exercise. This compound had previously been studied because of its potential as antimicrobial agent [19].

Concerning acylglycerides, glycerol ester isomers from C16:0, C18:0 and C20:0 (1-monopalmitoylglycerol, 2-monopalmitoylglycerol, 1-monostearoylglycerol, 2-monostearoylglycerol and 1-monoarachidoylglycerol) were detected in sweat, as well as the monoacylglycerol from isobutyric acid (1-monoisobutyroylglycerol). Some monoglycerides such as 1-monopalmitoylglycerol and 1-monostearoylglycerol had been previously identified in sweat [10]. Other fatty acid derivatives detected in sweat were fatty alcohols, mostly saturated (1-docecanol, 1-tetradecanol, 1-hexadecanol, 1-octadecanol), but also an unsaturated fatty alcohol (3-nonen-1-ol). Regarding unsaponifiable lipids, only one steroid (cholesta-3,5-diene), and two prenol lipids such as dehydroabietic acid (diterpene) and squalene (triterpene) were identified. Squalene, involved in the synthesis of cholesterol [20], had been previously identified in sweat [10].

Amino acids are other key group of metabolites identified in sweat, including essential (8) and no essential (9) amino acids, but also a few no proteinogenic amino acids (8). These last compounds play key roles as intermediates in metabolic pathways. Thus, aminoadipic and aminomalonic acids, citrulline, pyroglutamic acid and sarcosine play roles as intermediates in the formation of lysine, alanine, arginine, glutamic acid and glycine, while ornithine is involved in the urea cycle (urea was also detected). A neurotransmitter —γ-aminobutyric acid (GABA)— and 2-aminoheptanedioic acid were also detected. Most

of these amino acids or derivatives had previously been identified in sweat using different protocols [8,9].

Table 3. Compounds identified by GC–TOF/MS in active sweat classified by the chemical family.

Compound	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments	Family
Nicotine	10.89	$C_{10}H_{14}N_2$	54-11-5	$C_{10}H_{14}N_2$	133.0759 119.0611 84.0808	Alkaloids
Cotinine	15.01	$C_{10}H_{12}N_2O$	486-56-6	$C_{10}H_{12}N_2O$	176.0938 118.0640 98.0599	Alkaloids
Caffeine	16.52	$C_8H_{10}N_4O_2$	58-08-2	$C_8H_{10}N_4O_2$	194.0798 109.0624 67.0301	Alkaloids
Ethanolamine	9.36	C_2H_7NO	141-43-5	$C_{11}H_{31}NOSi_3$	174.1133 131.0884 117.0735	Alkanolamines
Diethanolamine	9.60	$C_4H_{11}NO_2$	111-42-2	$C_{10}H_{27}NO_2Si_2$	146.0987 130.0682 59.0310	Alkanolamines
	11.67			$C_{13}H_{35}NO_2Si_3$	218.1395 130.0682 56.0497	
Triethanolamine	13.99	$C_6H_{15}NO_3$	102-71-6	$C_{15}H_{39}NO_3Si_3$	262.1655 117.0714 59.0307	Alkanolamines
Valine	6.75	$C_5H_{11}NO_2$	72-18-4	$C_8H_{19}NO_2Si$	146.0648 72.0814 55.0547	Amino acids
	8.62			$C_{11}H_{27}NO_2Si_2$	246.1324 218.1110 203.0809	
Alanine	6.94	$C_3H_7NO_2$	56-41-7	$C_9H_{23}NO_2Si_2$	218.1013 190.1067 116.0885	Amino acids
Glycine	7.24	$C_2H_5NO_2$	56-40-6	$C_8H_{21}NO_2Si_2$	204.0876 176.0914 102.0735	Amino acids
	9.92			$C_{11}H_{29}NO_2Si_3$	276.1248 248.1303 174.1125	
Leucine	7.77	$C_6H_{13}NO_2$	61-90-5	$C_9H_{21}NO_2Si$	86.0964 75.0271 44.0497	Amino acids
Isoleucine	8.10	$C_6H_{13}NO_2$	73-32-5	$C_9H_{21}NO_2Si$	170.0994 86.0963 75.0271	Amino acids
	9.73			$C_{12}H_{29}NO_2Si_2$	260.1489 232.1535 158.1357	
Serine	9.24	$C_3H_7NO_3$	302-84-1	$C_9H_{23}NO_3Si_2$	234.0954 206.0477 193.0507	Amino acids
	10.64			$C_{12}H_{31}NO_3Si_3$	218.1033 204.1283 188.0924	

Continuation Table 3

Proline	9.81	$C_5H_9NO_2$	609-36-9	$C_{11}H_{25}NO_2Si_2$	216.1224 170.0620 142.1043	Amino acids
Threonine	10.98	$C_4H_9NO_3$	72-19-5	$C_{13}H_{33}NO_3Si_3$	291.1513 218.1342 117.0734	Amino acids
Sarcosine	11.19	$C_3H_7NO_2$	107-97-1	$C_9H_{23}NO_2Si_2$	190.1074 147.0659 116.0888	Amino acids
Aspartic acid	11.51	$C_4H_7NO_4$	617-45-8	$C_{10}H_{23}NO_4Si_2$	262.0921 172.0462 160.0787	Amino acids
	12.71			$C_{13}H_{31}NO_4Si_3$	232.1202 218.1042 100.0587	Amino acids
Aminomalonic acid	12.08	$C_3H_5NO_4$	1068-84-4	$C_{12}H_{29}NO_4Si_3$	320.1163 292.1216 218.1028	Amino acids
Pyroglutamic acid	12.48	$C_5H_7NO_3$	149-87-1	$C_8H_{15}NO_3Si$	106.0635 84.0445 56.0497	Amino acids
	12.74			$C_{11}H_{23}NO_3Si_2$	156.0863 93.5422 84.0436	Amino acids
γ-Aminobutyric acid	12.85	$C_4H_9NO_2$	56-12-2	$C_{13}H_{33}NO_2Si_3$	304.1577 174.1127 100.0217	Amino acids
α-Aminoadipic acid	13.05	$C_6H_{11}NO_4$	1118-90-7	$C_{15}H_{35}NO_4Si_3$	334.1635 260.1497 100.0218	Amino acids
2-Aminoheptanedioic acid	13.70	$C_7H_{13}NO_4$	627-76-9	$C_{16}H_{37}NO_4Si_3$	274.1660 218.1033 73.0470	Amino acids
Ornithine	13.86	$C_5H_{12}N_2O_2$	70-26-8	$C_{14}H_{36}N_2O_2Si_3$	348.2070 142.1039 115.0789	Amino acids
	16.10			$C_{17}H_{44}N_2O_2Si_4$	420.2496 147.1142 142.1060	
Glutamic acid	13.91	$C_5H_9NO_4$	56-86-9	$C_{14}H_{33}NO_4Si_3$	348.1465 246.1333 230.1019	Amino acids
Phenylalanine	14.01	$C_9H_{11}NO_2$	63-91-2	$C_{15}H_{27}NO_2Si_2$	294.1339 218.1021 192.1194	Amino acids
Asparagine	14.49	$C_4H_8N_2O_3$	70-47-3	$C_{13}H_{32}N_2O_3Si_3$	231.1341 188.0919 116.0842	Amino acids
Lysine	14.92	$C_6H_{14}N_2O_2$	56-87-1	$C_{15}H_{38}N_2O_2Si_3$	362.2214 230.1162 156.1187	Amino acids
	17.19			$C_{18}H_{46}N_2O_2Si_4$	317.2265 174.1133 156.1207	
Glutamine	15.63	$C_5H_{10}N_2O_3$	56-85-9	$C_{14}H_{34}N_2O_3Si_3$	245.1492 156.0844 75.0262	Amino acids
Citrulline	16.18	$C_6H_{13}N_3O_3$	372-75-8	$C_{18}H_{45}N_3O_3Si_4$	157.1156 142.1040 100.0217	Amino acids

Continuation Table 3

	18.97			$C_{15}H_{37}N_3O_3Si_3$	391.2138 100.0218 70.0656	
Histidine	17.16	$C_6H_9N_3O_2$	71-00-1	$C_{15}H_{33}N_3O_2Si_3$	254.1512 182.1105 154.0933	Amino acids
Tyrosine	17.36	$C_9H_{11}NO_3$	60-18-4	$C_{18}H_{35}NO_3Si_3$	382.1682 354.1731 280.1538	Amino acids
Tryptophan	19.57	$C_{11}H_{12}N_2O_2$	73-22-3	$C_{14}H_{20}N_2O_2Si$	159.0916 130.0660 75.0263	Amino acids
	19.78			$C_{17}H_{28}N_2O_2Si_2$	218.1024 130.0645 73.0464	
	19.89			$C_{17}H_{28}N_2O_2Si_2$	202.1046 130.0644 45.0153	
	19.96			$C_{20}H_{36}N_2O_2Si_3$	291.1496 218.1023 202.1045	
Indane	6.05	C_9H_{10}	496-11-7	C_9H_{10}	117.0694 91.0538 63.0146	Benzene derivatives
Benzoic acid	9.14	$C_7H_6O_2$	65-85-0	$C_{10}H_{14}O_2Si$	194.0739 179.0515 105.0325	Benzene derivatives (acids)
4-Acetyloxybenzoic acid	14.50	$C_9H_8O_4$	2345-34-8	$C_{12}H_{16}O_4Si$	210.0714 195.0488 135.0284	Benzene derivatives (acids)
4-Hydroxyphenyllactic acid	16.95	$C_9H_{10}O_4$	306-23-0	$C_{18}H_{34}O_4Si_3$	308.1252 293.1031 179.0884	Benzene derivatives (acids)
α-Keto-4-hydroxyben-zenepropanoic acid	17.04	$C_9H_8O_4$		$C_{16}H_{27}NO_4Si_2$	338.1240 277.1320 190.0689	Benzene derivatives (acids)
β-Ethenylben-zeneethanol	6.05	$C_{10}H_{12}O$	6052-63-7	$C_{10}H_{12}O$	117.0691 91.0534 51.0118	Benzene derivatives (alcohols)
α,α-Dimethylben-zenemethanol	6.76	$C_9H_{12}O$	617-94-7	$C_9H_{12}O$	121.0645 103.0529 43.0178	Benzene derivatives (alcohols)
Phenylethyl alcohol	7.19	$C_8H_{10}O$	60-12-8	$C_8H_{10}O$	91.0541 65.0384 51.0213	Benzene derivatives (alcohols)
Cathecol	10.08	$C_6H_6O_2$	120-80-9	$C_{12}H_{22}O_2Si_2$	254.1156 151.0219 73.0471	Benzene derivatives (alcohols)
3-Methylcathecol	11.06	$C_7H_8O_2$	488-17-5	$C_{13}H_{24}O_2Si_2$	268.1312 253.1061 180.0609	Benzene derivatives (alcohols)
2,4-Ditertbutylphenol	12.63	$C_{14}H_{22}O$	96-76-4	$C_{14}H_{22}O$	206.1074 191.1430 163.1114	Benzene derivatives (alcohols)
Pyrogallol	13.70	$C_6H_6O_3$	87-66-1	$C_{15}H_{30}O_3Si_3$	342.1490 239.0553 211.0599	Benzene derivatives (alcohols)
Triclosan	19.14	$C_{12}H_7Cl_3O_2$	3380-34-5	$C_{12}H_7Cl_3O_2$	252.9851 218.0133 145.9683	Benzene derivatives (clorinated)

Continuation Table 3

Isobutylparaben	13.01	$C_{11}H_{14}O_3$	4247-02-3	$C_{11}H_{14}O_3$	138.0311 121.0284 93.0358	Benzene derivatives (esters)
Carbamate	8.40	CH_3NO_2	463-77-4	$C_{10}H_{27}NO_2Si_3$	147.0659 131.0359 100.0217	Carbamic acids
Trehalose	24.09	$C_{12}H_{22}O_{11}$	6138-23-4	$C_{36}H_{86}O_{11}Si_8$	361.1689 243.1224 191.0900	Carbohydrates (disaccharides)
Erythrulose	14.56	$C_4H_8O_4$	496-55-9	$C_{16}H_{41}NO_4Si_4$	320.1512 117.0641 103.0472	Carbohydrates (monosaccharides)
Sorbitol	16.48	$C_6H_{12}O_5$	154-58-5	$C_{18}H_{44}O_5Si_4$	259.1165 217.1061 191.0894	Carbohydrates (monosaccharides)
Galactose	16.83	$C_6H_{12}O_6$	59-23-4	$C_{21}H_{52}O_6Si_5$	305.1384 265.1097 204.0998	Carbohydrates (monosaccharides)
	17.06			$C_{22}H_{55}NO_6Si_5$	319.1559 205.1053 160.0764	
Glucose	16.90	$C_6H_{12}O_6$	50-99-7	$C_{21}H_{52}O_6Si_5$	305.1388 265.1093 204.0998	Carbohydrates (monosaccharides)
	17.10			$C_{22}H_{55}NO_6Si_5$	319.1563 205.1065 160.0784	
Glyceric acid	10.22	$C_3H_6O_4$	473-81-4	$C_{12}H_{30}O_4Si_3$	307.1189 292.1329 189.0760	Carbohydrates (sugar acids)
Threonic acid	12.96	$C_4H_8O_5$	20246-26-8	$C_{16}H_{40}O_5Si_4$	292.1326 220.0931 205.1057	Carbohydrates (sugar acids)
Gluconic acid	17.92	$C_6H_{12}O_7$	526-95-4	$C_{24}H_{60}O_7Si_6$	333.1387 292.1350 117.0371	Carbohydrates (sugar acids)
Glycerol	9.43	$C_3H_8O_3$	56-81-5	$C_{12}H_{32}O_3Si_3$	293.1401 218.1127 205.1071	Carbohydrates (sugar alcohols)
Erythritol	12.53	$C_4H_{10}O_4$	149-32-6	$C_{16}H_{42}O_4Si_4$	217.1075 205.1066 117.0432	Carbohydrates (sugar alcohols)
Mannitol	17.29	$C_6H_{14}O_6$	69-65-8	$C_{24}H_{62}O_6Si_6$	319.1590 277.1472 217.1086	Carbohydrates (sugar alcohols)
Inositol	18.19	$C_6H_{12}O_6$	488-59-5	$C_{24}H_{60}O_6Si_6$	612.2920 305.1403 217.1059	Carbohydrates (sugar alcohols)
myo-Inositol	18.80		87-89-8	$C_{24}H_{60}O_6Si_6$	612.2926 305.1405 217.1060	
2,3-Butanediol	5.82	$C_4H_{10}O_2$	513-85-9	$C_{10}H_{26}O_2Si_2$	147.0653 133.0181 117.0720	Other alcohols and polyols
Propylene glycol	5.98	$C_3H_8O_2$	57-55-6	$C_9H_{24}O_2Si_2$	147.0662 117.0726 73.0469	Other alcohols and polyols
1,3-Butanediol	6.61	$C_4H_{10}O_2$	107-88-0	$C_{10}H_{26}O_2Si_2$	191.0911 117.0721 59.0310	Other alcohols and polyols

Continuation Table 3

Pinacol	7.75	$C_6H_{14}O_2$	76-09-5	$C_{12}H_{30}O_2Si_2$	131.0881 115.0561 73.0464	Other alcohols and polyols
1,2-Butanediol	8.05	$C_4H_{10}O_2$	584-03-2	$C_{10}H_{26}O_2Si_2$	131.0882 117.0714 73.0469	Other alcohols and polyols
Diethylene glycol	9.04	$C_4H_{10}O_3$	111-46-6	$C_{10}H_{26}O_3Si_2$	147.0658 117.0716 101.0409	Other alcohols and polyols
1-Methylene-3-methylbutane-1,3-diol	9.19	$C_6H_{12}O_2$		$C_{12}H_{28}O_2Si_2$	147.0655 131.0886 45.0155	Other alcohols and polyols
2-(2-Butoxy-ethoxy)ethanol	10.37	$C_8H_{18}O_3$	112-34-5	$C_{11}H_{26}O_3Si$	117.0713 101.0416 85.0643	Other alcohols and polyols
1,1,1-Tris(hydroxy-methyl)propane	11.06	$C_6H_{14}O_3$	77-99-6	$C_{15}H_{38}O_3Si_3$	191.0913 157.1036 129.0712	Other alcohols and polyols
Succinic acid	10.04	$C_4H_6O_4$	110-15-6	$C_{10}H_{22}O_4Si_2$	247.0818 147.0668 75.0264	Dicarboxylic acids
Itaconic acid	10.41	$C_5H_6O_4$	97-65-4	$C_{11}H_{22}O_4Si_2$	259.0812 215.0913 147.0655	Dicarboxylic acids
Citraconic acid	10.50	$C_5H_6O_4$	498-23-7	$C_{11}H_{22}O_4Si_2$	259.0818 184.0558 97.0460	Dicarboxylic acids
Fumaric acid	10.54	$C_4H_4O_4$	110-17-8	$C_{10}H_{20}O_4Si_2$	245.0665 217.0707 147.0656	Dicarboxylic acids
Glutaric acid	11.27	$C_5H_8O_4$	110-94-1	$C_{11}H_{24}O_4Si_2$	261.0968 233.1024 129.0438	Dicarboxylic acids
Malic acid	12.30	$C_4H_6O_5$	617-48-1	$C_{13}H_{30}O_5Si_3$	265.1114 233.1022 101.0408	Dicarboxylic acids
Adipic acid	12.56	$C_6H_{10}O_4$	124-04-9	$C_{12}H_{26}O_4Si_2$	172.0903 141.0731 111.0439	Dicarboxylic acids
Azelaic acid	15.91	$C_9H_{16}O_4$	123-99-9	$C_{15}H_{32}O_4Si_2$	317.1576 217.1069 117.0382	Dicarboxylic acids
Aconitic acid	15.35	$C_6H_6O_6$	499-12-7	$C_{15}H_{30}O_6Si_3$	375.1119 229.1073 211.0436	Tricarboxylic acids
Pyruvic acid	6.10	$C_3H_4O_3$	127-17-3	$C_7H_{15}NO_3Si$	174.0582 158.0625 130.0646	Carboxylic acids (keto acids)
α-Ketoglutaric acid	13.38	$C_5H_6O_5$	328-50-7	$C_7H_{12}O_4$	129.0548 100.0518 59.0491	Carboxylic acids (keto acids)
Lactic acid	5.79	$C_3H_6O_3$	50-21-5	$C_6H_{14}O_3Si$	119.0543 101.0416 75.0272	Carboxylic acids (hydroxy acids)
	6.25			$C_9H_{22}O_3Si_2$	191.0952 117.0823 45.0187	
Glycolic acid	6.51	$C_2H_4O_3$	79-14-1	$C_8H_{20}O_3Si_2$	147.0658 133.0432 66.0211	Carboxylic acids (hydroxy acids)

Continuation Table 3

1-Undecyne	7.03	$C_{11}H_{20}$	2243-98-3	$C_{11}H_{20}$	109.0990 95.0845 81.0689	Hydrocarbons
Caproic acid	6.51	$C_6H_{12}O_2$	142-62-1	$C_9H_{20}O_2Si$	173.0995 117.0375 75.0266	Lipids (fatty acids and conjugates-medium chain fatty acids)
Caprylic acid	9.36	$C_8H_{16}O_2$	124-07-2	$C_{11}H_{24}O_2Si$	201.1304 117.0450 75.0262	Lipids (fatty acids and conjugates-medium chain fatty acids)
Pelargonic acid	10.69	$C_9H_{18}O_2$	112-05-0	$C_{12}H_{26}O_2Si$	215.1465 129.0367 117.0370	Lipids (fatty acids and conjugates-medium chain fatty acids)
Capric acid	11.97	$C_{10}H_{20}O_2$	334-48-5	$C_{13}H_{28}O_2Si$	229.1619 129.0371 75.0261	Lipids (fatty acids and conjugates-medium chain fatty acids)
Lauric acid	14.33	$C_{12}H_{24}O_2$	143-07-7	$C_{15}H_{32}O_2Si$	257.1934 117.0369 75.0263	Lipids (fatty acids and conjugates-medium chain fatty acids)
Tridecylic acid	15.43	$C_{13}H_{26}O_2$	638-53-9	$C_{16}H_{34}O_2Si$	271.2093 117.0376 75.0266	Lipids (fatty acids and conjugates-long chain fatty acids)
Mirystoleic acid	16.25	$C_{14}H_{26}O_2$	544-64-9	$C_{17}H_{34}O_2Si$	283.2080 145.0690 129.0370	Lipids (fatty acids and conjugates-long chain fatty acids)
Myristic acid	16.48	$C_{14}H_{28}O_2$	544-63-8	$C_{17}H_{36}O_2Si$	285.2251 145.0681 117.0370	Lipids (fatty acids and conjugates-long chain fatty acids)
Pentadecylic acid	17.48	$C_{15}H_{30}O_2$	1002-84-2	$C_{18}H_{38}O_2Si$	299.2406 129.0372 117.0377	Lipids (fatty acids and conjugates-long chain fatty acids)
Palmitic acid	17.59	$C_{16}H_{32}O_2$	57-10-3	$C_{16}H_{32}O_2$	227.1997 129.0891 73.0279	Lipids (fatty acids and conjugates-long chain fatty acids)
	18.44			$C_{19}H_{40}O_2Si$	313.2541 269.1921 145.0674	
Palmitoleic acid	18.19	$C_{16}H_{30}O_2$	373-49-9	$C_{19}H_{38}O_2Si$	311.2403 129.0370 185.0990	Lipids (fatty acids and conjugates-long chain fatty acids)
10-Heptadecenoic acid	19.12	$C_{17}H_{32}O$	29743-97-3	$C_{20}H_{40}O_2Si$	325.2551 250.2277 185.0997	Lipids (fatty acids and conjugates-long chain fatty acids)
Margaric acid	19.36	$C_{17}H_{34}O_2$	506-12-7	$C_{20}H_{42}O_2Si$	327.2710 145.0682 117.0365	Lipids (fatty acids and conjugates-long chain fatty acids)
Arachidonic acid	19.83	$C_{20}H_{32}O_2$	506-32-1	$C_{23}H_{40}O_2Si$	129.0432 117.0382 55.0541	Lipids (fatty acids and conjugates-long chain fatty acids)

Continuation Table 3

Linoleic acid	19.83	$C_{18}H_{32}O_2$	60-33-3	$C_{21}H_{40}O_2Si$	262.2289 117.0403 67.0535	Lipids (fatty acids and conjugates-long chain fatty acids)
Oleic acid	20.01	$C_{18}H_{34}O_2$	112-80-1	$C_{21}H_{42}O_2Si$	339.2723 145.0679 117.0369	Lipids (fatty acids and conjugates-long chain fatty acids)
Stearic acid	19.51	$C_{18}H_{36}O_2$	57-11-4	$C_{18}H_{36}O_2$	284.2706 129.0908 73.0281	Lipids (fatty acids and conjugates-long chain fatty acids)
	20.24			$C_{21}H_{44}O_2Si$	341.2854 129.0360 117.0360	
Arachidic acid	21.90	$C_{20}H_{40}O_2$	506-30-9	$C_{23}H_{48}O_2Si$	369.3185 145.0698 129.0380	Lipids (fatty acids and conjugates-long chain fatty acids)
Lignoceric acid	24.88	$C_{24}H_{48}O_2$	557-59-5	$C_{27}H_{56}O_2Si$	425.3817 129.0387 117.0379	Lipids (fatty acids and conjugates-very long chain fatty acids)
Hexacosanoic acid	26.41	$C_{26}H_{52}O_2$	506-46-7	$C_{29}H_{60}O_2Si$	453.4126 129.0377 117.0369	Lipids (fatty acids and conjugates-very long chain fatty acids)
Dipropylacetic acid	9.36	$C_8H_{16}O_2$	99-66-1	$C_{11}H_{24}O_2Si$	201.1305 174.1128 117.0435	Lipids (methyl-branched fatty acids)
2-Hydroxybutyric acid	7.25	$C_4H_8O_3$	565-70-8	$C_{10}H_{24}O_3Si_2$	147.0659 131.0886 73.0470	Lipids (hydroxy fatty acids)
3-Hydroxybutyric acid	7.81	$C_4H_8O_3$	300-85-6	$C_{10}H_{24}O_3Si_2$	233.1021 191.0913 117.0713	Lipids (hydroxy fatty acids)
3-Hydroxyisovaleric acid	7.85	$C_5H_{10}O_3$	17407-56-6	$C_{11}H_{26}O_3Si_2$	147.0650 131.0876 115.0560	Lipids (hydroxy fatty acids)
2-Hydroxyisocaproic acid	8.91	$C_6H_{12}O_3$	498-36-2	$C_{12}H_{28}O_3Si_2$	177.0744 159.1194 103.0548	Lipids (hydroxy fatty acids)
5-Hydroxy-*n*-valeric acid	10.29	$C_5H_{10}O_3$	13392-69-3	$C_{11}H_{26}O_3Si_2$	247.1180 231.0882 147.0658	Lipids (hydroxy fatty acids)
Erucylamide	24.51	$C_{22}H_{43}NO$	112-84-5	$C_{22}H_{43}NO$	337.3338 126.0916 72.0440	Lipids (fatty amides)
Mono(2-ethylhexyl)-adipate	21.50	$C_{14}H_{26}O_4$	4337-65-9	$C_{14}H_{26}O_4$	129.0549 111.0440 83.0487	Lipids (fatty acid esters)
1-Monoisobu-tyroylglycerol	12.08	$C_7H_{14}O_4$		$C_{13}H_{30}O_4Si_2$	203.1091 117.0407 59.0310	Lipids (acylglycerides)
2-Monopalmito-ylglycerol	22.76	$C_{19}H_{38}O_4$	23470-00-0	$C_{25}H_{54}O_4Si_2$	313.2552 191.0010 147.0655	Lipids (acylglycerides)
1-Monopalmitoylglycerol	23.02	$C_{19}H_{38}O_4$	26657-96-5	$C_{25}H_{54}O_4Si_2$	371.2961 239.2359 203.0900	Lipids (acylglycerides)

Continuation Table 3

2-Monostearoylglycerol	24.20	$C_{21}H_{42}O_4$	621-61-4	$C_{27}H_{58}O_4Si_2$	341.2863 218.1136 191.0851	Lipids (acylglycerides)
1-Monostearoylglycerol	24.45	$C_{21}H_{42}O_4$	123-94-4	$C_{27}H_{58}O_4Si_2$	399.3293 341.2868 267.2691	Lipids (acylglycerides)
1-Monoarachi-doylglycerol	25.86	$C_{23}H_{46}O_4$	50906-68-8	$C_{29}H_{62}O_4Si_2$	427.3606 411.4017 295.2997	Lipids (acylglycerides)
Dehydroabietic acid	21.54	$C_{20}H_{28}O_2$	740-19-8	$C_{23}H_{36}O_2Si$	357.2222 239.1791 197.1315	Lipids (prenol lipids-diterpenes)
Squalene	24.76	$C_{30}H_{50}$	111-02-4	$C_{30}H_{50}$	410.3907 121.0994 81.0686	Lipids (prenol lipids-triterpenes)
Hedione	14.35	$C_{13}H_{22}O_3$	24851-98-7	$C_{13}H_{22}O_3$	226.1566 97.0623 83.0479	Lipids (lineolic acids and derivatives-jasmonic acids)
Cholesta-3,5-diene	25.47	$C_{27}H_{44}$	747-90-0	$C_{27}H_{44}$	368.3437 247.2412 147.1141	Lipids (steroids and steroids derivatives)
3-Nonen-1-ol	7.03	$C_9H_{18}O$	10340-23-5	$C_9H_{18}O$	95.0848 81.0694 67.0540	Lipids (fatty alcohols)
1-Dodecanol	13.35	$C_{12}H_{26}O$	112-53-8	$C_{15}H_{34}OSi$	243.2136 129.0699 97.1002	Lipids (fatty alcohols)
1-Tetradecanol	15.58	$C_{14}H_{30}O$	112-72-1	$C_{17}H_{38}OSi$	271.2444 111.1161 97.1007	Lipids (fatty alcohols)
1-Hexadecanol	17.62	$C_{16}H_{34}O$	36653-82-4	$C_{19}H_{42}OSi$	299.2764 97.1001 55.0538	Lipids (fatty alcohols)
1-Octadecanol	19.47	$C_{18}H_{38}O$	112-92-5	$C_{21}H_{46}OSi$	327.3084 241.1977 97.1007	Lipids (fatty alcohols)
Phosphate	9.42	PO_4^{3-}	7664-38-2	$C_9H_{27}O_4PSi_3$	299.0712 211.0008 133.0156	No metal oxoanionic compounds
2-Furoic acid	7.46	$C_5H_4O_3$	88-14-2	$C_8H_{12}O_3Si$	169.0314 125.0416 95.0193	Organoheterocyclic compounds
Uracil	10.34	$C_4H_4N_2O_2$	66-22-8	$C_{10}H_{20}N_2O_2Si_2$	241.0818 99.0350 45.0157	Organoheterocyclic compounds
Uridine	15.12	$C_9H_{12}N_2O_6$	58-96-8	$C_{18}H_{36}N_2O_6Si_3$	259.1164 217.1063 103.0487	Organoheterocyclic compounds
Urocanic acid	15.63	$C_6H_6N_2O_2$	7699-35-6	$C_{12}H_{22}N_2O_2Si_2$	267.0975 193.0784 120.0308	Organoheterocyclic compounds
Xanthine	18.13	$C_5H_4N_4O_2$	69-89-6	$C_{14}H_{28}N_4O_2Si_3$	353.1281 294.1324 238.0843	Organoheterocyclic compounds
Uric acid	18.89	$C_5H_4N_4O_3$	69-93-2	$C_{17}H_{36}N_4O_3Si_4$	456.1680 441.1635 73.0467	Organoheterocyclic compounds
Urea	8.10	CH_4N_2O	57-13-6	$C_{10}H_{28}N_2OSi_3$	276.1501 261.1273 245.0959	Ureas

Continuation Table 3

	9.00			$C_7H_{20}N_2OSi_2$	189.0879 171.0775 147.0663	
Borate	9.64	BO_3^{3-}	10043-35-3	$C_9H_{27}BO_3Si_3$	263.1140 221.0861 175.0447	Exogenous
Methylparaben	11.92	$C_8H_8O_3$	99-76-3	$C_8H_8O_3$	152.0470 121.0292 93.0337	Exogenous
	12.46			$C_{11}H_{16}O_3Si$	224.0863 209.0622 193.0666	
Propylparaben	12.84	$C_{10}H_{12}O_3$	94-13-3	$C_{10}H_{12}O_3$	138.0314 121.0290 93.0337	Exogenous

Other no polar chemical family found in sweat was that of aromatic homomonocyclic compounds, mainly benzene derivatives. It is worth mentioning among them benzene acid derivatives such as benzoic acid, 4-acetyloxybenzoic acid, 4-hydroxyphenyllactic acid and α-keto-4-hydroxybenzenepropanoic acid, a benzene ester derivative (isobutylparaben), and benzene alcohol derivatives such as β-ethenylbenzeneethanol, α,α-dimethylbenzenemethanol, phenylethyl alcohol, cathecol, 3-methylcathecol, 2,4-ditertbutylphenol and pyrogallol. Other benzene derivatives detected in sweat were triclosan (clorinated derivative) and indane. It is remarkable the presence of exogenous compounds such as triclosan and parabens from cosmetics. For example, triclosan is present in shampoos, deodorants and toothpastes, as it has shown effectiveness in reducing and controlling bacterial contamination on the hands [21]; while parabens are commonly used as preservatives in cosmetics [22].

Short carboxylic acids, which are also representative of crucial biochemical pathways [23], were other group of metabolites detected in sweat. It is worth mentioning the presence in this group of compounds related to the Krebs' cycle as aconitic acid, succinic, α-ketoglutaric, fumaric, pyruvic and malic acids. Compounds produced in the body during the metabolism of some amino acids such as lysine and tryptophan are glutaric acid and other dicarboxylic acids such as itaconic, citraconic, adipic and azelaic acids, which were also identified in sweat. Carboxylic acid derivatives identified were hydroxy acids such as lactic and glycolic acids. Some dicarboxylic acids such as azelaic acid had previously been identified in sweat [9].

Identified sugars included monosaccharides (erythrulose, sorbitol, galactose and glucose), a disaccharide (trehalose), sugar alcohols (glycerol, erythritol, mannitol and

inositol) and sugar acids (glyceric, threonic and gluconic acids), some of them previously identified in sweat, such as glucose and inositol [7]. Other identified alcohols and polyols were propylene glycol, diethylene glycol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, pinacol, 1-methylene-3-methylbutane-1,3-diol, 2-(2-butoxyethoxy)ethanol and 1,1,1-tris(hydroxymethyl)propane.

Organo heterocyclic compounds detected in sweat were uracil, uridine (uracil nucleoside), uric acid (product of metabolic breakdown of purine nucleotides), urocanic acid (intermediate in the catabolism of histidine), xanthine (an intermediate in the degradation of adenosine monophosphate to uric acid) and 2-furoic acid (a metabolite that appears in urine after exposition to furfural and is also naturally present in coffee). Also phosphate was identified in sweat.

Other identified families were alkaloids and alkanoamines. Identified alkaloids were mainly stimulants such as caffeine and nicotine. Furthermore, cotinine, the predominant metabolite of nicotine, was detected in sweat. Regarding alkanolamines, ethanolamine, diethanolamine and triethanolamine were also found.

3.4. Comparison of active sweat collected after moderate exercise and passive sweat

In this study, active sweat collected after moderate exercise was compared with passive sweat from the same volunteers collected after induction by pilocarpine. In the first case, sweat was collected from three different parts of the body to increase the sampling representativeness. For passive sampling, sweat was only collected from the arm because the Macroduct system limits the sampling area.

A previous study confirmed deproteination as the preferred sample preparation strategy for analysis of passive sweat collected with the Macroduct system [10]. This result was validated here by testing the different sample preparation approaches evaluated in this research. Therefore, the protocols that have provided better results in each of the cases, LLE with dichoromethane for active sweat after moderate exercise and deproteination for passive sweat were qualitatively and semiquantitatively compared. The two pools of sweat (active and passive) were analyzed in triplicate. In both cases, derivatization was carried out prior to GC–MS analysis. Fig. 5 shows the Venn diagram comparing the number of

compounds tentatively identified in the dichloromethane extract of active sweat and the deproteinated passive sweat collected by pilocarpine induction. The list of identified compounds in these analytical samples is shown in Table S1. The analysis of active sweat allowed detection of 118 compounds, while that of passive sweat provided 87. One fact to be emphasized is that 92% of the compounds detected in passive sweat were also detected in active sweat. Most of the compounds exclusively detected in active sweat were identified as lipids (42%), most of them long and very long-chain fatty acids, amino acids (15%) and sugars (15%), among others.

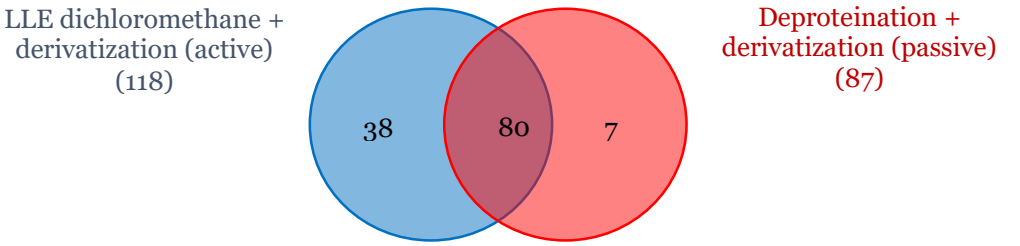

Fig. 5. Venn diagram comparing the number of compounds tentatively identified in dichloromethane extract of active sweat and deproteinated passive sweat collected after pilocarpine induction. (Identification of each compound in a given extract was validated if its peak area was above 10% of the sum of its peak areas reported in the three extracts).

A heat map was built to compare qualitative and quantitatively compositional differences between active and passive sweat. The resulting heat map, illustrated in Fig. 6, shows metabolomic differences between sweat collected after exercise and that collected after chemical induction.

Concerning the family of compounds, benzenoids exhibited a similar concentration range in the two protocols, except for methylparaben, which was more concentrated in passive sweat because the Macroduct discs used for sweating induction contain this compound. Carboxylic acids such as dicarboxylic acids, keto acids and hydroxy acids also exhibited a similar concentration in sweat collected by the two protocols. Also organoheterocyclic compounds and essential amino acids were found in the two pools at similar concentration.

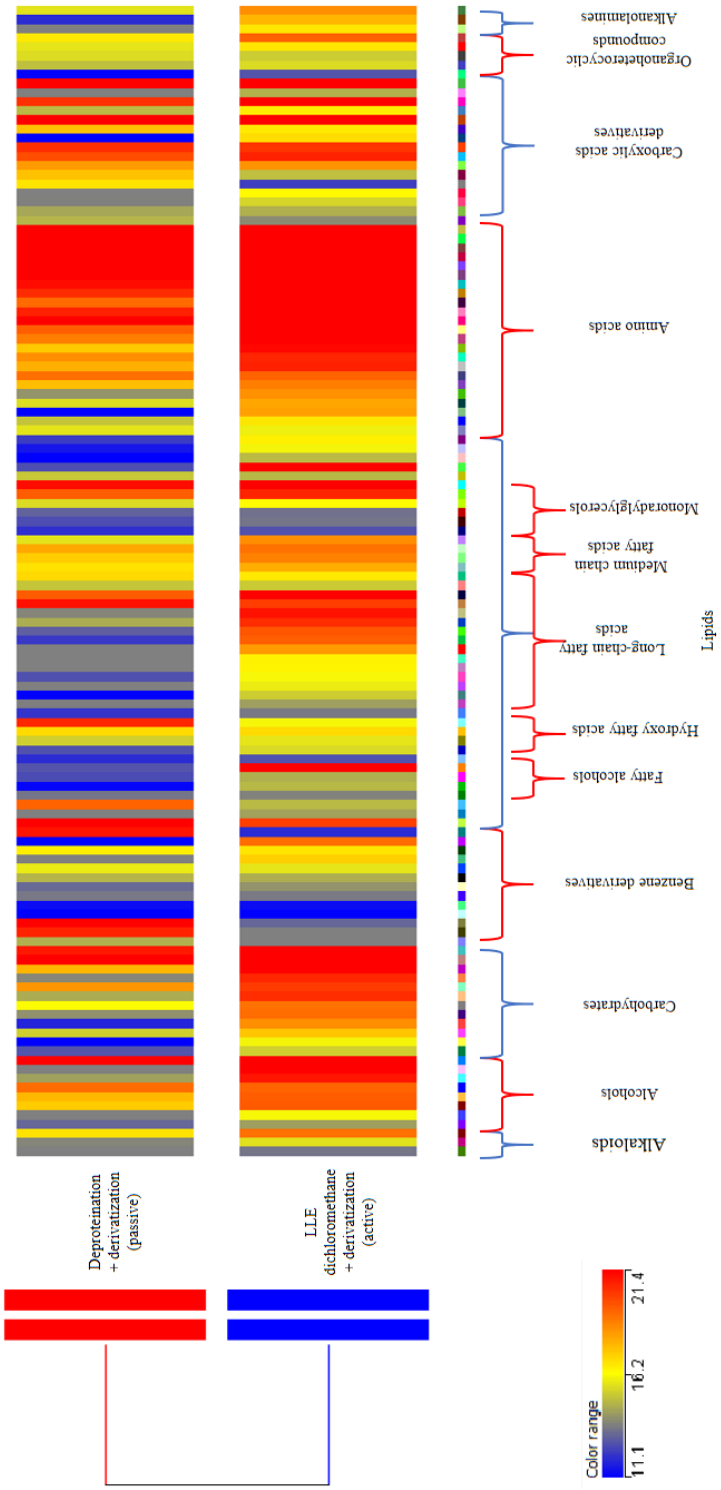

Fig. 6. Heat map comparing relative concentrations of compounds tentatively identified in sweat collected after both exercise and chemical induction with pilocarpine. Color coding indicates differences in normalized concentration for each metabolite.

The most significant differences were found in alcohols, carbohydrates, lipids and no proteinogenic amino acids, as shown in Table S1, which reports higher concentration in sweat after moderate exercise.

These metabolic differences can be explained by the stimulation of perspiration since active sweat involves the response to the practice of the moderate exercise and, therefore, sweat collected under these conditions should reflect a new metabolic state. Thus, alcohols such as diols (1,2-butanediol, 1,3-butanediol, 2,3-butanediol and pinacol) and glycols (diethylene and propylene glycol) were clearly found at higher concentration in active sweat as compared to passive sweat —see Fig. 7A.

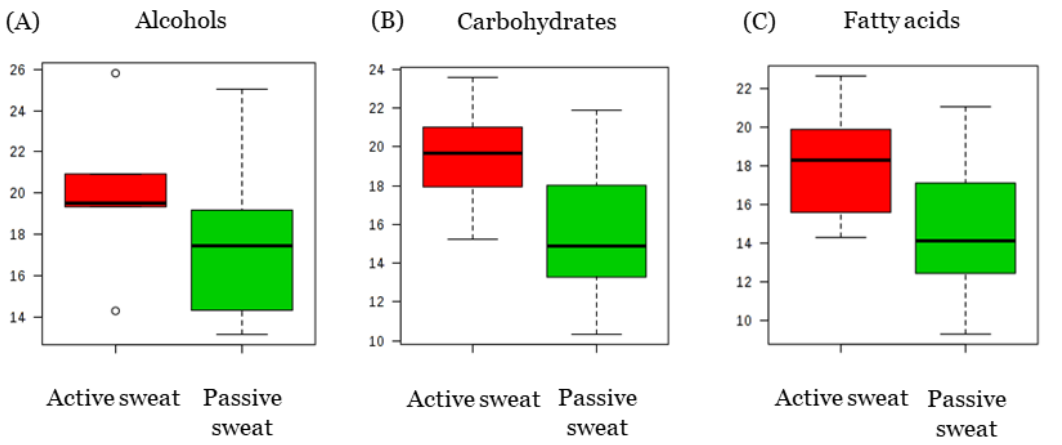

Fig. 7. The box-and-whisker plots of three families of metabolites found at different relative concentrations in active sweat and passive sweat. The bottom and top of the boxes represent the 25th and 75th percentile, respectively. The top and bottom bars represent the entire stretch of the data points for the subjects, except the extreme points, which are indicated with circles (o). The hyphen indicates the median value. The *y* axis represents the normalized peak area of the metabolites.

Carbohydrates, particularly monosaccharides and sugar alcohols, were specially excreted in sweat after practicing moderate exercise. Glucose levels in sweat have been correlated to blood glucose as a no invasive proposal to control glycaemia in diabetic patients [24]. However, the levels of glucose in sweat as well as those of other metabolites such as lactic acid or gluconic acid can provide information about the response of the energetic metabolism to exercise. Concentration differences for carbohydrates commonly

detected in the two pools are clear in Fig. 7B. Fatty acids and squalene also gave an enriched concentration profile in active sweat as compared to passive sweat, in which most fatty acids were detected at lower concentrations. Only stearic acid and caproic acid presented higher levels in passive sweat —see Fig. 7C. The difference in no polar compounds is one of the most representative changes found in sweat collected after the two stimulation protocols.

No proteinogenic amino acids were other family of metabolites found at higher concentration in active sweat, which could be explained by an activation of the synthesis and metabolism of amino acids in individuals after practicing exercise, but also could be associated to the contribution of the skin microbiota [25].

Other compounds such as α-keto-4-hydroxybenzenepropanoic acid, mono(2-ethyhexyl)adipate and 5-hydroxy-n-valeric acid exhibited higher concentration in sweat collected after induction with pilocarpine. However, no special trends were observed for any family of compounds as occurred with active sweat. Finally, it is worth mentioning that passive sweat was only sampled from the arm of volunteers, which is a limitation of the Macroduct device, while active sweat was collected from three different areas of the body (forehead, chest and back). Therefore, the sampling of different parts of the body can increase representativeness based on results provided by other studies that have reported differences in sweat composition according to the sampling area [7,26]

4. Conclusions

Sample preparation for untargeted analysis of active sweat collected after moderate exercise has been evaluated by testing LLE with different extractants, protein precipitation and derivatization prior to GC–MS analysis. Among the tested protocols, LLE with dichloromethane with subsequent methoxymation plus silylation was the best option to obtain a representative snapshot of active sweat due to the detection of no polar compounds, scarcely detected in previous studies developed with passive sweat. Compositional differences in passive and active sweat collected from the same volunteers were found, offering the latter an enriched concentration of some important families of compounds such as fatty acids, alcohols, carbohydrates and no proteinogenic amino acids. The differences in concentration can be explained by the sampling protocol since sweat is stimulated in a different manner prior to collection of active and passive sweat.

Additionally, the sampling location was not the same due to operational restrictions of the Macroduct device.

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Supplementary material

Table S1. Compounds identified by GC–TOF/MS in dichloromethane extract of active and depreoteinated sweat collected after chemical induction (passive) after derivatization in both cases.

Compound	RT (min)	Formula	Family	LLE dichloro-methane + derivatization (active sweat)	Deproteination + derivatization (passive sweat)	*p*-Value[a]
Cotinine	15.01	$C_{10}H_{12}N_2O$	Alkaloids	✓	✓	0.0017**
Caffeine	16.52	$C_8H_{10}N_4O_2$	Alkaloids	✓	✓	0.0006**
Ethanolamine	9.36	C_2H_7NO	Alkanolamines	✓	✓	0.0013**
Diethanolamine	9.60	$C_4H_{11}NO_2$	Alkanolamines	✓		0.0000**
	11.67			✓		
Triethanolamine	13.99	$C_6H_{15}NO_3$	Alkanolamines	✓	✓	0.0001**
Valine	*6.75*	$C_5H_{11}NO_2$	Amino acids	✓		0.0355*
	8.62			✓	✓	
Alanine	6.94	$C_3H_7NO_2$	Amino acids	✓	✓	0.1288
Glycine	*7.24*	$C_2H_5NO_2$	Amino acids	✓	✓	0.6787
	9.92			✓	✓	
Leucine	7.77	$C_6H_{13}NO_2$	Amino acids	✓	✓	0.0598
Isoleucine	*8.10*	$C_6H_{13}NO_2$	Amino acids	✓	✓	0.0883
	9.73			✓	✓	
Serine	*9.24*	$C_3H_7NO_3$	Amino acids	✓		0.0165*
	10.64			✓	✓	
Proline	9.81	$C_5H_9NO_2$	Amino acids	✓	✓	0.3035
Threonine	10.98	$C_4H_9NO_3$	Amino acids	✓	✓	0.0026**
Sarcosine	11.19	$C_3H_7NO_2$	Amino acids	✓	✓	0.0000**
Aspartic acid	12.71	$C_4H_7NO_4$	Amino acids	✓	✓	0.0010**
Aminomalonic acid	12.08	$C_3H_5NO_4$	Amino acids	✓	✓	0.0013**
Pyroglutamic acid	*12.48*	$C_5H_7NO_3$	Amino acids	✓	✓	0.0126*
	12.74		Amino acids	✓	✓	

Continuation Table S1

γ-Aminobutyric acid	12.85	$C_4H_9NO_2$	Amino acids	✓	✓	0.0010**
α-Aminoadipic acid	13.05	$C_6H_{11}NO_4$	Amino acids	✓	✓	0.5767
2-Aminohep-tanedioic acid	13.70	$C_7H_{13}NO_4$	Amino acids	✓	✓	0.0001**
Ornithine	*13.86*	$C_5H_{12}N_2O_2$	Amino acids	✓	✓	0.0003**
	16.10			✓	✓	
Glutamic acid	13.91	$C_5H_9NO_4$	Amino acids	✓	✓	0.0011**
Phenylalanine	14.01	$C_9H_{11}NO_2$	Amino acids	✓	✓	0.0003**
Asparagine	14.49	$C_4H_8N_2O_3$	Amino acids	✓	✓	0.2935
Lysine	*14.92*	$C_6H_{14}N_2O_2$	Amino acids	✓	✓	0.0011**
	17.19			✓	✓	
Glutamine	15.63	$C_5H_{10}N_2O_3$	Amino acids	✓	✓	0.8569
Citrulline	*16.18*	$C_6H_{13}N_3O_3$	Amino acids	✓		0.0000**
	18.97			✓	✓	0.1118
Histidine	17.16	$C_6H_9N_3O_2$	Amino acids	✓	✓	0.0255*
Tyrosine	17.36	$C_9H_{11}NO_3$	Amino acids	✓	✓	0.0009**
Tryptophan	19.57	$C_{11}H_{12}N_2$-O_2	Amino acids	✓	✓	0.0795
	19.78			✓	✓	
	19.89			✓	✓	
	19.96			✓	✓	
Indane	6.05	C_9H_{10}	Benzene derivatives	✓	✓	0.5058
Benzoic acid	9.14	$C_7H_6O_2$	Benzene derivatives (acids)	✓	✓	0.0438*
4-Acetyloxyben-zoic acid	14.50	$C_9H_8O_4$	Benzene derivatives (acids)	✓	✓	$2\ 10^{-5}$**
4-Hydroxy-phenyllactic acid	16.95	$C_9H_{10}O_4$	Benzene derivatives (acids)	✓	✓	0.0003**
α-Keto-4-hydroxyben-zenepropanoic acid	17.04	$C_9H_8O_4$	Benzene derivatives (acids)	✓		0.0000**
β-Ethenylben-zeneethanol	6.05	$C_{10}H_{12}O$	Benzene derivatives (alcohols)	✓	✓	0.2490
Cathecol	10.08	$C_6H_6O_2$	Benzene derivatives (alcohols)	✓	✓	0.8819
2,4-Ditert-butylphenol	12.63	$C_{14}H_{22}O$	Benzene derivatives (alcohols)	✓	✓	0.8381

Continuation Table S1

Pyrogallol	13.70	$C_6H_6O_3$	Benzene derivatives (alcohols)	✓	✓	0.0045**
Carbamate	8.40	CH_3NO_2	Carbamic acids	✓	✓	0.0000**
Trehalose	24.09	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)	✓	✓	0.0002**
Erythrulose	14.56	$C_4H_8O_4$	Carbohydrates (monosaccharides)	✓	✓	0.0001**
Sorbitol	16.48	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)	✓	✓	0.0001**
Galactose	*16.83*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓	0.0005**
	17.06			✓	✓	
Glucose	*16.90*	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓	0.0017**
	17.10			✓	✓	
Glyceric acid	10.22	$C_3H_6O_4$	Carbohydrates (sugar acids)	✓	✓	0.0017**
Threonic acid	12.96	$C_4H_8O_5$	Carbohydrates (sugar acids)	✓	✓	0.0003**
Gluconic acid	17.92	$C_6H_{12}O_7$	Carbohydrates (sugar acids)	✓	✓	0.0001**
Glycerol	9.43	$C_3H_8O_3$	Carbohydrates (sugar alcohols)	✓	✓	0.9214
Erythritol	12.53	$C_4H_{10}O_4$	Carbohydrates (sugar alcohols)	✓	✓	0.0033**
Mannitol	17.29	$C_6H_{14}O_6$	Carbohydrates (sugar alcohols)	✓	✓	0.0002**
Inositol	*18.19*	$C_6H_{12}O_6$	Carbohydrates (sugar alcohols)	✓	✓	0.0005**
myo-Inositol	18.80			✓	✓	0.0002**
2,3-Butanediol	5.82	$C_4H_{10}O_2$	Other alcohols and polyols	✓	✓	0.0020**
Propylene glycol	5.98	$C_3H_8O_2$	Other alcohols and polyols	✓	✓	0.0018**
1,3-Butanediol	6.61	$C_4H_{10}O_2$	Other alcohols and polyols	✓	✓	$4{\cdot}10^{-5}$**
Pinacol	7.75	$C_6H_{14}O_2$	Other alcohols and polyols	✓	✓	0.1656
1,2-Butanediol	8.05	$C_4H_{10}O_2$	Other alcohols and polyols	✓		0.0000**
Diethylene glycol	9.04	$C_4H_{10}O_3$	Other alcohols and polyols	✓	✓	0.0039**
1-Methylene-3-methylbutane-1,3-diol	9.19	$C_6H_{12}O_2$	Other alcohols and polyols	✓		0.0000**
2-(2-Butoxy-ethoxy)ethanol	10.37	$C_8H_{18}O_3$	Other alcohols and polyols	✓	✓	0.1599
Succinic acid	10.04	$C_4H_6O_4$	Dicarboxylic acids	✓	✓	0.3953
Itaconic acid	10.41	$C_5H_6O_4$	Dicarboxylic acids	✓	✓	0.0000**

Continuation Table S1

Citraconic acid	10.50	$C_5H_6O_4$	Dicarboxylic acids	✓	✓	0.1334
Fumaric acid	10.54	$C_4H_4O_4$	Dicarboxylic acids	✓	✓	0.3023
Glutaric acid	11.27	$C_5H_8O_4$	Dicarboxylic acids	✓	✓	0.6470
Malic acid	12.30	$C_4H_6O_5$	Dicarboxylic acids	✓	✓	0.0038**
Adipic acid	12.56	$C_6H_{10}O_4$	Dicarboxylic acids	✓	✓	0.0574
Azelaic acid	15.91	$C_9H_{16}O_4$	Dicarboxylic acids	✓		0.0000**
Aconitic acid	15.35	$C_6H_6O_6$	Tricarboxylic acids	✓		0.0000**
Pyruvic acid	6.10	$C_3H_4O_3$	Carboxylic acids (keto acids)	✓	✓	0.0009**
α-Ketoglutaric acid	13.38	$C_5H_6O_5$	Carboxylic acids (keto acids)	✓	✓	0.0091**
Lactic acid	6.25	$C_3H_6O_3$	Carboxylic acids (hydroxy acids)	✓	✓	$3 \cdot 10^{-7}$**
Glycolic acid	6.51	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)	✓	✓	0.0139*
Caproic acid	6.51	$C_6H_{12}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	0.0930
Caprylic acid	9.36	$C_8H_{16}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	0.1043
Pelargonic acid	10.69	$C_9H_{18}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	0.1104
Capric acid	11.97	$C_{10}H_{20}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	0.1791
Lauric acid	14.33	$C_{12}H_{24}O_2$	Lipids (fatty acids and conjugates-medium chain fatty acids)	✓	✓	0.0032**
Tridecylic acid	15.43	$C_{13}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0009**
Mirystoleic acid	16.25	$C_{14}H_{26}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓		0.0000**
Myristic acid	16.48	$C_{14}H_{28}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0001**
Pentadecylic acid	17.48	$C_{15}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0001**

Continuation Table S1

Palmitic acid	18.44	$C_{16}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0095**
Palmitoleic acid	18.19	$C_{16}H_{30}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0001**
10-Heptade-cenoic acid	19.12	$C_{17}H_{32}O$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓		0.0000**
Margaric acid	19.36	$C_{17}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0024**
Arachidonic acid	19.83	$C_{20}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓		0.0000**
Linoleic acid	19.83	$C_{18}H_{32}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓		0.0000**
Oleic acid	20.01	$C_{18}H_{34}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.0051**
Stearic acid	20.24	$C_{18}H_{36}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.2156
Arachidic acid	21.90	$C_{20}H_{40}O_2$	Lipids (fatty acids and conjugates-long chain fatty acids)	✓	✓	0.1478
Lignoceric acid	24.88	$C_{24}H_{48}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)	✓	✓	0.0016**
Hexacosanoic acid	26.41	$C_{26}H_{52}O_2$	Lipids (fatty acids and conjugates-very long chain fatty acids)	✓	✓	0.0015**
Dipropylacetic acid	9.36	$C_8H_{16}O_2$	Lipids (methyl-branched fatty acids)	✓	✓	0.0013**
2-Hydroxy-butyric acid	7.25	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓	0.0009**
3-Hydroxy-butyric acid	7.81	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓	0.9700
3-Hydroxy-isovaleric acid	7.85	$C_5H_{10}O_3$	Lipids (hydroxy fatty acids)	✓	✓	0.0137*
5-Hydroxy-n-valeric acid	10.29	$C_5H_{10}O_3$	Lipids (hydroxy fatty acids)	✓	✓	0.0557
Erucylamide	24.51	$C_{22}H_{43}NO$	Lipids (fatty amides)	✓	✓	0.0119*
Mono(2-ethylhexyl)-adipate	21.50	$C_{14}H_{26}O_4$	Lipids (fatty acid esters)	✓	✓	0.0244*

Continuation Table S1

1-Monoiso-butyroylglycerol	12.08	$C_7H_{14}O_4$	Lipids (acylglycerides)	✓	✓	0.0012**
2-Monopalmito-ylglycerol	22.76	$C_{19}H_{38}O_4$	Lipids (acylglycerides)	✓	✓	0.3886
1-Monopalmito-ylglycerol	23.02	$C_{19}H_{38}O_4$	Lipids (acylglycerides)	✓	✓	0.0057**
2-Mono-stearoylglycerol	24.20	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓	0.0162*
1-Monos tearoylglycerol	24.45	$C_{21}H_{42}O_4$	Lipids (acylglycerides)	✓	✓	0.0050**
1-Monoarachi-doylglycerol	25.86	$C_{23}H_{46}O_4$	Lipids (acylglycerides)	✓	✓	0.0012**
Dehydroabietic acid	21.54	$C_{20}H_{28}O_2$	Lipids (prenol lipids-diterpenes)	✓	✓	0.3188
Squalene	24.76	$C_{30}H_{50}$	Lipids (prenol lipids-triterpenes)	✓	✓	0.0002**
Hedione	14.35	$C_{13}H_{22}O_3$	Lipids (lineolic acids and derivatives-jasmonic acids)	✓	✓	0.0441*
Cholesta-3,5-diene	25.47	$C_{27}H_{44}$	Lipids (steroids and steroids derivatives)	✓		0.0000**
1-Dodecanol	13.35	$C_{12}H_{26}O$	Lipids (fatty alcohols)	✓	✓	0.0005**
1-Tetradecanol	15.58	$C_{14}H_{30}O$	Lipids (fatty alcohols)	✓	✓	0.0006**
1-Hexadecanol	17.62	$C_{16}H_{34}O$	Lipids (fatty alcohols)	✓	✓	0.1957
1-Octadecanol	19.47	$C_{18}H_{38}O$	Lipids (fatty alcohols)	✓	✓	0.0002**
Phosphate	9.42	PO_4^{3-}	No metal oxoanionic compounds	✓	✓	0.0023*
2-Furoic acid	7.46	$C_5H_4O_3$	Organoheterocy-clic compounds	✓	✓	0.1325
Uracil	10.34	$C_4H_4N_2O_2$	Organoheterocy-clic compounds	✓	✓	0.8341
Uridine	15.12	$C_9H_{12}N_2O_6$	Organoheterocy-clic compounds	✓	✓	0.0130*
Urocanic acid	15.63	$C_6H_6N_2O_2$	Organoheterocy-clic compounds	✓	✓	0.0040**
Xanthine	18.13	$C_5H_4N_4O_2$	Alkaloids	✓		0.0000**
Uric acid	18.89	$C_5H_4N_4O_3$	Organoheterocy-clic compounds	✓	✓	0.9858
Urea	*8.10*	CH_4N_2O	Ureas	✓	✓	0.0000**
	9.00			✓	✓	
Borate	9.64	BO_3^{3-}	Exogenous		✓	0.0000**
Methylparaben	12.46	$C_8H_8O_3$	Exogenous	✓	✓	$4{\cdot}10^{-5}$**
Pilocarpine	18.65	$C_{11}H_{16}N_2O_2$	Exogenous		✓	0.0000**

[a] An unpaired *t*-test statistical analysis was used to evaluate the significance of the compounds identified in dichloromethane extract of active and depreoteinated sweat collected after chemical induction to discriminate**;** * *p*-Value between 0.01 and 0.05**;** ** *p*-Value < 0.01.

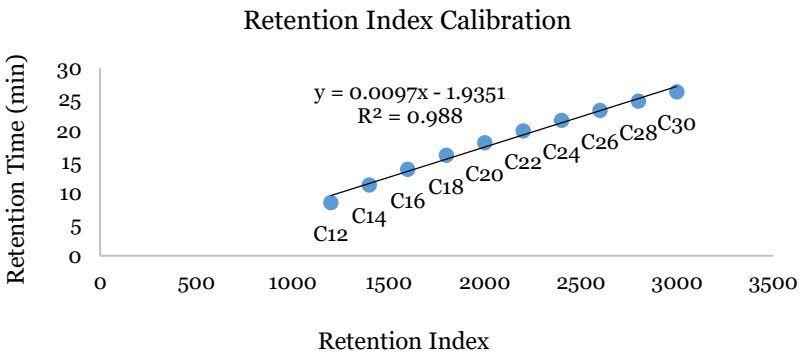

Fig. S1. RI calibration line obtained by analysis of the alkane standard mixture.

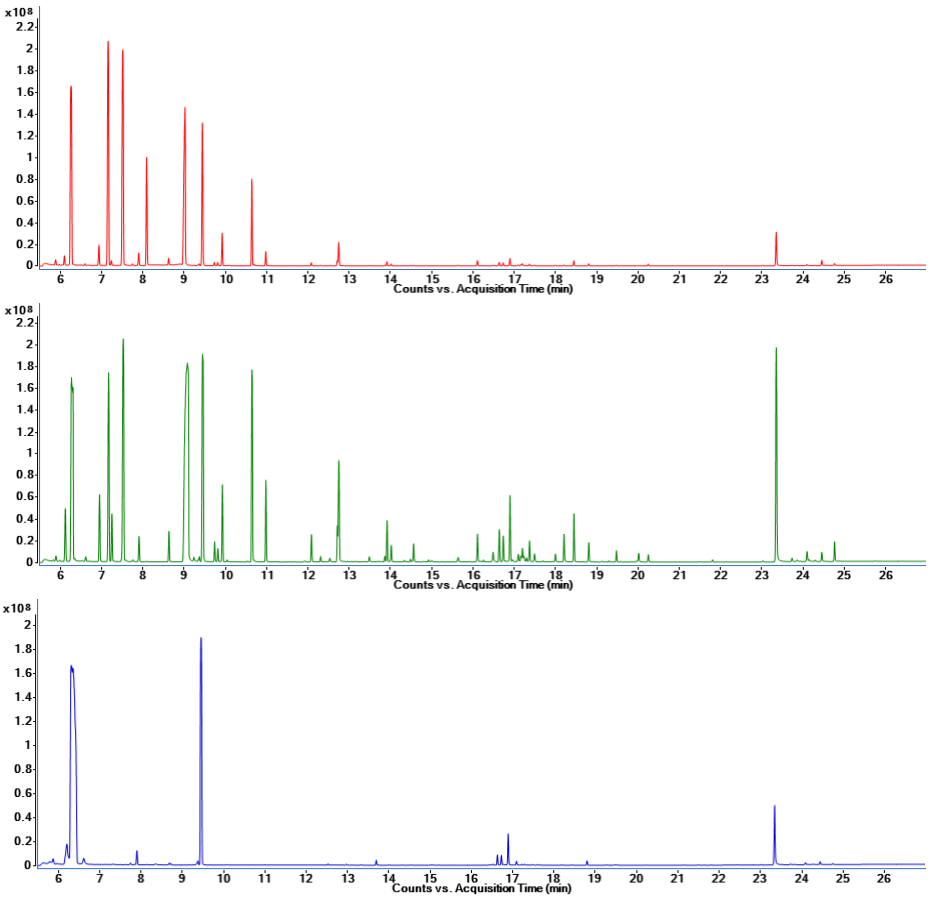

Fig. S2. Base peak chromatograms provided by analysis of the derivatized extracts of active sweat obtained with three extractants, ethyl acetate (red), dichloromethane (green) and hexane (blue).

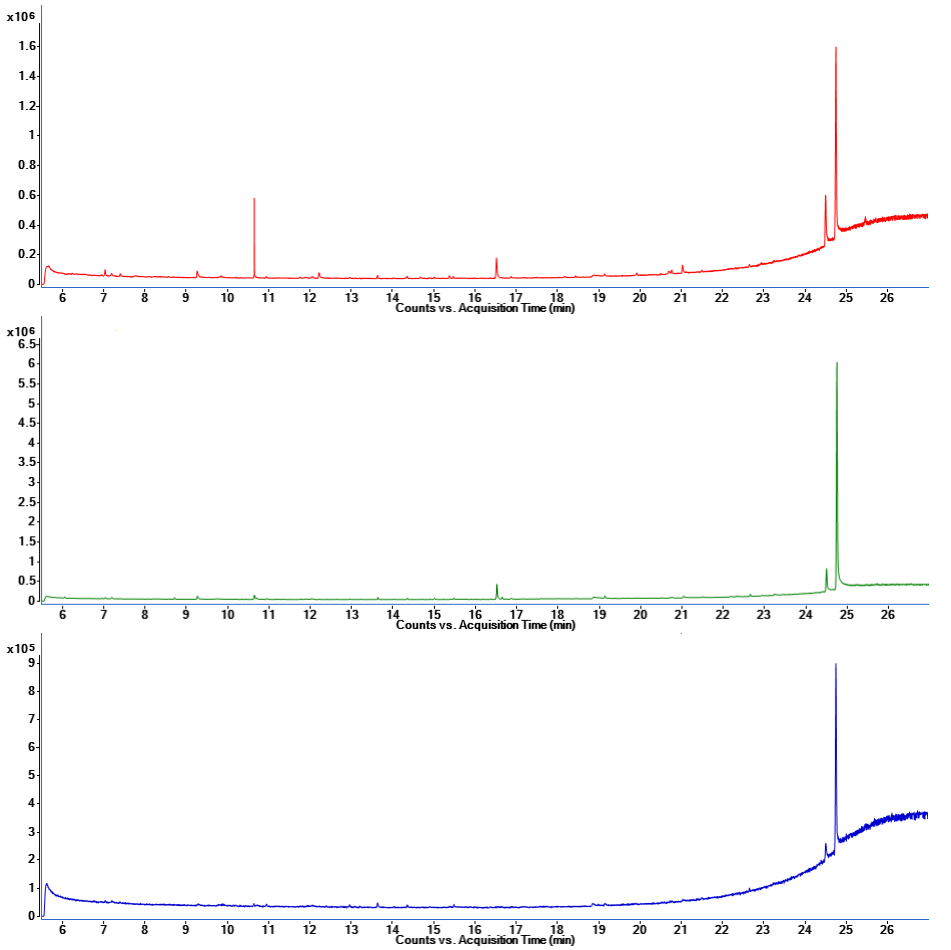

Fig. S3. Base peak chromatograms provided by direct analysis of the extracts of active sweat obtained with three extractants, ethyl acetate (red), dichloromethane (green) and hexane (blue).

Chapter IX

Dry sweat as sample for metabolomics analysis

Dry sweat as sample for metabolomics analysis

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Dry sweat as sample for metabolomics analysis

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Abstract

Sweat is gaining popularity in clinical metabolomics as this biofluid is no invasively sampled and its composition is modified by several pathologies. There is a lack of standardized strategies for collection of human sweat. Most studies have been carried out with fresh sweat collected after stimulation. A promising and simple alternative is sampling dry sweat by a solid support impregnated with a suited solvent. This research was aimed at comparing the metabolomics coverage provided by dry sweat collected by two solid supports (gauzes and filter papers) impregnated with different solvents. The dissolved dry sweat was analyzed by a dual approach: gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Among the tested sampling strategies, filter paper impregnated with 1:1 (v/v) ethanol–phosphate buffer resulted the combination providing the highest metabolomics coverage (tentative identification of 175 compounds). Dry and fresh sweat were compared by using pools from the same individuals to evaluate compositional differences. Families of metabolites such as carnitines, sphingolipids and N-acyl-amino acids, among others, were exclusively identified in dry sweat. Comparison of both samples allowed concluding that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds. As most of the identified metabolites are involved in key biochemical pathways, this study opens interesting possibilities to the use of dry sweat as a source of metabolite markers for specific disorders. Sampling of dry sweat could provide a standardized approach for collection of this biofluid, thus overcoming the variability limitations of fresh sweat.

Keywords: dry sweat metabolome, metabolomics analysis, sampling protocol, gas chromatography, liquid chromatography, mass spectrometry.

1. Introduction

Sweat is a biofluid produced by eccrine and procrine glands located in the epidermis [1]. This slightly acidic biofluid (pH range 4.0–7.0) is composed mainly of water (99%) containing electrolytes, small molecules, proteins, peptides, metal ions, and xenobiotics, among others. These substances are stored in the sweat glands, secreted and, finally, transported to the epidermis surface [1]. Sweat excretion is affected by several factors such as ambient temperature, relative humidity, area of the body skin, hormonal imbalances, overactive thyroid gland and the sympathetic nervous system, and certain foods and medications [2]. Sweating is also naturally increased by nervousness, exercise, stress and nausea. With sampling purposes, sweating must be stimulated by physical exercise or at rest, usually by heat or iontophoretic induction with chemicals such as pilocarpine, to obtain enough volume of this biofluid for analysis [3].

Sweat collection is a step to which scant attention has been paid, despite the collection format seems to be responsible for uncontrolled variability sources in the analysis of this biofluid. Most sweat analyses have been carried using fresh sweat [3–6], which has been used as such or treated after a drying process [7,8]. Fresh sweat can be collected by occlusive or no occlusive covering devices. In the former case, sweat has been mainly sampled on a bandage of filter paper, cotton, gauze or towel [9], and frequently associated to sweat suppression or skin irritation. Additionally, this sampling is typically lengthened to hours [10]. Other alternative involved the use of hydrogel micropatches as sampling device of skin excretions [11–13]. Hydrogel micropatches have been used to profile skin metabolites with identification of eight compounds [11], to monitor two drugs on skin surface [14] and to screen psoriasis-related skin metabolites [12]. Despite these limitations, novel wearable digital devices based on an adhesive radio-frequency sensor bandage (patch) have been reported. These devices can establish a close contact with human skin, a distinct advantage for chronological monitoring of biomarkers in sweat [15–19]. As an example, a commercial radio-frequency identification chip has been adapted with minimum changes to allow sensing Na^+ in sweat by potentiometric measurements and surface temperature. All these measurements are read by an Android smartphone app with 96% accuracy at 50 mM Na^+ [16].

On the other hand, devices for no occlusive sampling of fresh sweat are typically based on capillarity or condensation, the latter involving an adhesive layer on a thin transparent film, which is in contact with a rectangular absorbent pad for sweat collection

[9]. A comprehensive study has been recently published, in which sweat was collected over 24-h periods using a no occlusive patch adhered to different skin zones [20]. The authors used differential chemical isotope labeling (CIL) and liquid chromatography–mass spectrometry (LC–MS) to map the metabolomic profile of the samples, which resulted in the tentative identification of 84 metabolites by using MS/MS information [20]. Concerning capillarity collection, Kutyshenko *et al.* developed a device with special pipettes and reverse capillaries for collection of small sweat volumes [4]. Commercial devices for capillarity collection of sweat such as the Macroduct®, based on iontophoresis with pilocarpine, are widely used for diagnostic of cystic fibrosis [21].

Most of the wide variety of fresh sweat samplers require to wear the device during the sweating process, which is tedious. For this reason, novel procedures for sweat collection are desirable. One alternative is collection of dry sweat, a concept that should not be confused with those applications based on sampling of fresh sweat, then dried for analysis. One example of this is the study developed by Sikirzhytski *et al.*, who dried fresh sweat on a microscopy slide prior to obtaining Raman spectra dominated by the contribution of lactic acid, urea and amino acids [7]. In one other application, fresh sweat was spotted onto collection paper containing butylated hydroxytoluene for solvent evaporation prior to determination of fatty acids by gas chromatography with flame ionization detection (GC–FID) [8].

A particular case specific for volatile organic compounds is the use of divinylbenzene/carboxen/polydimethylsiloxane 'Stableflex' fibers. The fiber came in contact with the skin for 30 min through a glass funnel. After collecting the volatiles, the SPME fiber was inserted into the injector of the GC–MS to desorb them [22].

Dry sweat can be collected after sweating stimulation by using a solid support to transfer sweat components from the skin. This protocol could be proposed to standardize the sampling process due to its simplicity [8]. For this purpose, the necessity for a well-established protocol for collection of dry sweat seems to be essential to characterize its composition. The aim of the present study was to compare different supports–solvents for sampling dry sweat by compositional analysis using untargeted approaches based on gas chromatography–time of flight/mass spectrometry (GC–TOF/MS) and liquid chromatography–quadrupole time of flight tandem mass spectrometry (LC–QTOF MS/MS). Fresh sweat was also analyzed to evaluate the detection coverage of metabolites in the different collection formats.

2. Experimental section

2.1. Reagents

Reagent grade ethanol from Scharlab (Barcelona, Spain), anhydrous potassium hydrogen phosphate from Panreac (Barcelona, Spain) and Optima™ LC–MS grade acetic acid from Thermo Fisher Scientific (Pittsburgh, PA, USA) were used for dissolution of dry sweat components.

Optima™ LC–MS grade formic acid (FA) and acetonitrile (ACN) from Thermo Fisher Scientific, and LC–MS grade methanol and GC grade dichloromethane from Sigma–Aldrich (St. Louis, MO, USA) were used for sample preparation. FA and ACN were also used to prepare the chromatographic mobile phases for LC–MS/MS analysis. Deionized water (18 mΩ·cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the aqueous chromatographic mobile phases and all other aqueous solutions.

Pyridine and methoxyamine hydrochloride from Sigma–Aldrich were used as solvent and reagent, respectively, for methoxymation. Bis-(trimethylsilyl) fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were used as silylation agents in the derivatization step. MS-grade perfluorotri-*n*-butylamine (PFTBA) from Agilent Technologies (Santa Clara, CA, USA) was used for daily mass calibration. A standard mixture containing ten linear alkanes from C10 to C40 designed for performance tests in GC from Sigma–Aldrich was used to establish the retention index (RI) calibration model. LC–grade *n*-hexane and acetone from Scharlab were used for washing the injection syringe. Standards of D-galactose, D-glucose and myo-inositol from Sigma–Aldrich were used to prepare standards for confirmation of sugars identification.

2.2. Instruments and apparatus

A vortex shaker from IKA (Wilmington, NC, USA), a Legend Micro 21R microcentrifuge from Thermo Fisher Scientific, a Concentrator plus™ from Eppendorf (Hamburg, Germany), and a ThermoMixer C, also from Eppendorf, were used in different steps of sample preparation.

An Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with a dual electrospray ionization (ESI) source was used. This ESI device enables to spray simultaneously a mass reference solution that calibrates continuously detected m/z ratios.

An Agilent 7890A Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with electron impact (EI) source was also used for analysis. MassHunter LC–QTOF Acquisition software (version B.08, Agilent Technologies) and MassHunter GC–QTOF Acquisition software (version B.07, Agilent Technologies) were used to control data acquisition and set the parameters for optimum operation. The analytical samples were monitored in high resolution mode both in LC–MS/MS and GC–MS analyses.

2.3. Sweat collection

Sweat samples were kindly donated by six healthy volunteers (3 men and 3 women) —all of them gave informed consent for sweat sampling. No restrictions about diet or age were considered. The use of deodorants, perfumes and cosmetics was excluded at least one day prior to sweat collection. The sampled skin area was cleaned with ethanol and distilled water before sweat stimulation. Sweat samples were collected from a given area of the back of about 100 cm^2. Previously, the donors were subjected to a moderate exercise session walking for 30 min at around 35 ºC ambient temperature.

Fresh and dry sweat were sampled simultaneously from similar back areas on each volunteer. Fresh sweat was collected directly from the skin by a glass Pasteur pipette with a latex bulb (supplied by Scharlab), then transferred to plastic micro Eppendorf tubes and stored at −80 °C until sample preparation. A total sweat volume of at least 100 μL was collected per sampling. On the other hand, dry sweat was collected after sweating stimulation by a gauze (10 cm×10 cm) from Difasan (Barcelona, Spain) or an ashless filter paper (10 cm×10 cm) from Filalbet (Barcelona, Spain), which were previously humidified with 250 μL of ethanol, pH 7 phosphate buffer solution (PBS) or an 1:1 (v/v) ethanol–PBS mixture. The wet supports were gently rubbed on the selected skin area, transferred to Falcon plastic tubes and stored at −80 °C until sample preparation. Sampling did not cause skin irritation during the process or on subsequent days.

2.4. Sample treatment

Different experimental protocols were used for treatment of fresh and dry sweat samples before LC–MS/MS and GC–MS analysis.

2.4.1 Sample treatment for fresh sweat

For LC–MS/MS analysis one aliquot of fresh sweat (100 µL) was deproteinized by vortexing with 100 µL of 50:50 methanol–ACN in a glass insert for 5 min at room temperature; then kept on ice for 5 min and centrifuged for 5 min at 10000×*g*. The liquid phase was isolated and located into a new glass insert, evaporated to dryness and reconstituted by 25 µL of 0.1% FA in water, as previously optimized Calderón-Santiago *et al.* [6].

For GC–MS analysis fresh sweat samples were deproteinized, evaporated to dryness and reconstituted by 25 µL of 0.1% FA in water. Then, 25 µL of each reconstituted sample was vortexed with 150 µL of dichloromethane in a glass insert at room temperature for 5 min and centrifuged for 5 min at 10000×*g*. Then, the organic phase was isolated and located into a new glass insert, evaporated to dryness and reconstituted by 20 µL of 40 mg/mL methoxyamine hydrochloride in pyridine. After adding the methoxymation solution, the mixture was shaken and maintained at 30 °C for 90 min. For subsequent silylation, 90 µL of a 100:1 BSTFA–TMCS mixture was added, shaken for 30 s and maintained at 37 °C for 30 min. All samples were cooled at room temperature until analysis. This sample preparation protocol was used in a previous study prior to analyzing sweat collected after moderate exercise [23].

2.4.2 Sample treatment for dry sweat

Humid gauzes or filter papers with dry sweat were vortexed for 5 min with 750 µL of the same solvent used for wetting it, and then centrifuged for 5 min at 10000×*g*. The liquid phase was filtered through a 0.45 µm pore size membrane filter from Agilent Technologies, located into a new glass vial, and evaporated to dryness. Aliquots for LC–MS/MS analysis were reconstituted with 25 µL of 0.1% FA in 50:50 water–methanol. On the other hand, aliquots for GC–MS analysis were reconstituted with 25 µL of 0.1% FA in water. Then, 25 µL of the reconstituted samples was vortexed with 150 µL of

dichloromethane in a glass insert at room temperature for 5 min and centrifuged for 5 min at 10000×*g*. The organic phase was isolated and located into a new glass insert, evaporated to dryness and reconstituted by 20 μL of 40 mg/mL methoxyamine hydrochloride in pyridine. After adding the methoxymation solution, the mixture was shaken and maintained at 30 °C for 90 min. Subsequent silylation involved addition of 90 μL of a 100:1 BSTFA–TMCS mixture, shaking for 30 s and keeping at 37 °C for 30 min. All samples were cooled at room temperature for subsequent analysis. Contamination from gauze and/or filter paper was discarded by analysis of water introduced in them as blank, which was subjected to the same protocols. In all cases, the samples were prepared in triplicate.

2.5. LC–QTOF MS/MS analysis

LC separation and QTOF detection were used for analysis of dry and fresh sweat samples. Chromatographic separation was performed using a C18 reverse-phase analytical column (Mediterranean, 50 mm×4.6 mm i.d., 3 μm particle size) from Teknokroma (Barcelona, Spain), which was thermostated at 25 ºC. The mobile phases were water (phase A) and ACN (phase B) both with 0.1% FA as ionization agent. The LC pump was programmed at a flow rate of 0.8 mL/min with the following elution gradient: 3% phase B as initial mobile phase was kept constant from min 0 to 1; then, the percentage of phase B was increased from 3 to 80% from min 1 to 10.5, and finally from 80 to 100% of phase B from min 10.5 to 11.5. A post-time of 5 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 3 μL and the injector needle was washed ten times between injections with 70% methanol. Furthermore, the needle seat back was flushed for 15 s at a flow rate of 4 mL/min with 70% methanol to avoid cross-contamination. The parameters of the ESI source, operating in negative and positive ionization modes, were as follows: the capillary and fragmentor voltage were set at ± 3.5 kV and 175 V, respectively; N2 in the nebulizer flowed at 40 psi; the flow rate and temperature of the N2 as drying gas were 8 L/min and 350 ºC, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. MS and MS/MS data were collected in both polarities separately using the centroid mode at a rate of 1 spectrum/s in the extended dynamic range mode (2 GHz). Accurate mass spectra in MS scan and MS/MS mode were acquired within the *m/z* range 60–1100. The instrument gave typical resolution 15000 FWHM (full width at half maximum) at *m/z* 118.0862 and 30000 FWHM at *m/z* 922.0098. To assure the desired mass accuracy of recorded ions, continuous

internal calibration was performed during analyses by using the signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H,1H,3Htetrafluoro-propoxy) phosphazine or HP-921] in the positive ionization mode; while in negative ionization mode ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.0007 (formate adduct of HP-921) were used. The samples were injected in auto-MS/MS acquisition mode to obtain information on the product ion of the relevant compounds. The number of precursors selected per cycle was set at 2, with an exclusion window of 0.3 min after two consecutive selections of the same precursor. The collision energy 20 eV was used to obtain the fragmentation information for each entity. At least three replicates of each analytical sample were analyzed to improve the quality of the results.

2.6. GC–TOF/MS analysis

GC–TOF/MS analyses were performed by EI ionization mode at 70 eV and controlled by MassHunter Acquisition B.06. Chromatographic separation was carried out with a fused silica DB-5MS-UI 30 m×0.25 mm i.d×0.25 μm film thickness capillary column. The GC oven temperature program started at 60 °C (1 min held), followed by a temperature ramp of 10 °C/min to final 300 °C (2 min held). A post-run time was programmed for 4 min up to 310 °C to assure complete elution of the injected sample. One μL of sample was injected using pulsed split injection (1:10 split ratio) at 250 °C (the data in the Fiehn library were obtained with this injection mode, and the sensitivity of the proposed method is enough for using this injection mode). Ultrapure grade helium was used as carrier gas at 0.9 mL/min. The transfer line, ion source and quadrupole temperatures were set at 280, 300 and 200 °C, respectively. A solvent delay of 5.50 min was used to prevent damage of the ion source filament. The TOF detector was operated at 5 spectra/s in the mass range *m/z* 40–750 and the resolution was 8500 FWHM at *m/z* 501.9706. Daily mass calibration was performed with PFTBA.

2.7. LC–QTOF MS/MS data processing and statistical analysis

Details including extraction of molecular features (MFs), tentative identification and integration of all peaks are provided as Supplementary material.

2.8. GC–TOF/MS data processing and statistical analysis

Details including deconvolution of chromatograms to obtain a list of MFs, tentative identification of compounds by the National Institute of Standards and Technology (NIST) database (supported on MS spectrum and retention index, Supplementary Fig. 1) and Fiehn database [24], and integration of all metabolites found are provided as Supplementary material.

3. Results and discussion

3.1. Influence of impregnation solvent on the collection of dry sweat

Gauze and filter paper were selected as supports for collection of dry sweat thanks to their simplicity and low price. A requirement is that they should be humidified with a solvent to solubilize dry sweat components from the skin. In this study, ethanol, PBS and 1:1 (v/v) ethanol–PBS were compared. Ethanol and PBS are characterized by medium and high polarity, respectively, while ethanol–PBS allows testing the effect of an intermediate polarity solvent. Additionally, the three solvents present low-toxicity and miscibility with water. Dry sweat samples from the six volunteers were collected on gauze and papers using independently the three solutions (6 volunteers × 2 supports × 3 solvents), and treated as detailed in Section 2.4. The analytical samples thus obtained were analyzed in triplicate by GC–TOF/MS and LC–QTOF MS/MS. Supplementary Fig. 2 shows the base peak chromatograms (BPCs) provided by analysis of sweat samples collected on filter paper (A) and gauze (B) using the two platforms. The number of chromatographic peaks increased when the ethanol–PBS mixture impregnated the two solid supports as compared to the use of ethanol or PBS independently. This increase in detection capability was then estimated as the number of tentatively identified metabolites in dry sweat by combination of GC–MS and LC–MS/MS analyses. The result is illustrated in the Venn diagrams of Fig. 1.

Sweat samples collected by filter paper impregnated with ethanol–PBS enabled the tentative identification of 156 compounds, while collection with the gauze using the same solvent composition led to the identification of 163 compounds. Both collection devices behaved similarly in terms of identified compounds. Samples in ethanol or PBS led to the

identification of around 83–91% of the sweat components detected in ethanol–PBS mixture using both paper and gauze as supports.

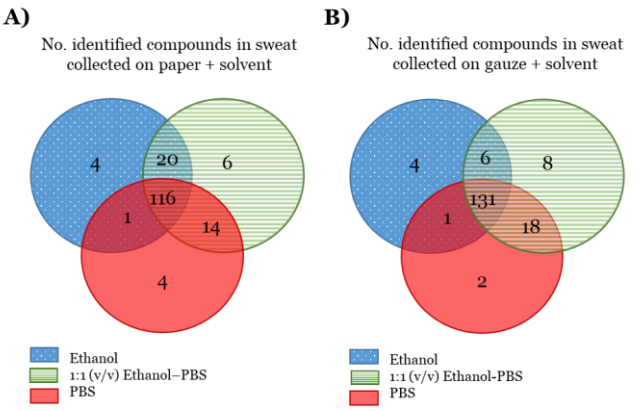

Fig. 1. Venn diagrams comparing the number of compounds tentatively identified by GC–TOF/MS and LC–QTOF MS/MS in dry sweat collected by the two supports and the three impregnation solutions.

The ethanol–PBS solution also offered the best performance in quantitative terms. The detection capability for ethanol–PBS solution was calculated for each pair ethanol–PBS/ethanol and ethanol–PBS/PBS by using the following expressions:

$$\text{Detection capability (\%)} = \frac{Peak\ area\ for\ compound\ X\ detected\ in\ ethanol\text{–PBS}}{Peak\ area\ for\ compound\ X\ detected\ in\ ethanol} \times 100$$

$$\text{and detection capability (\%)} = \frac{Peak\ area\ for\ compound\ X\ detected\ in\ ethanol\text{–PBS}}{Peak\ area\ for\ compound\ X\ detected\ in\ PBS} \times 100$$

Fig. 2 (A and B) shows the bar diagrams by plotting the number of compounds identified as a function of the detection capability using paper as support. As can be seen, most compounds reported a detection capability above 100% when ethanol–PBS was used as solution mixture. However, 16% of the metabolites —mainly lipids such as fatty acyls and glycerolipids— were detected with a higher efficiency when ethanol was used as solvent. On the other hand, 23% of compounds (mainly carboxylic acids such as hydroxy acids) were more efficiently detected in the PBS buffer.

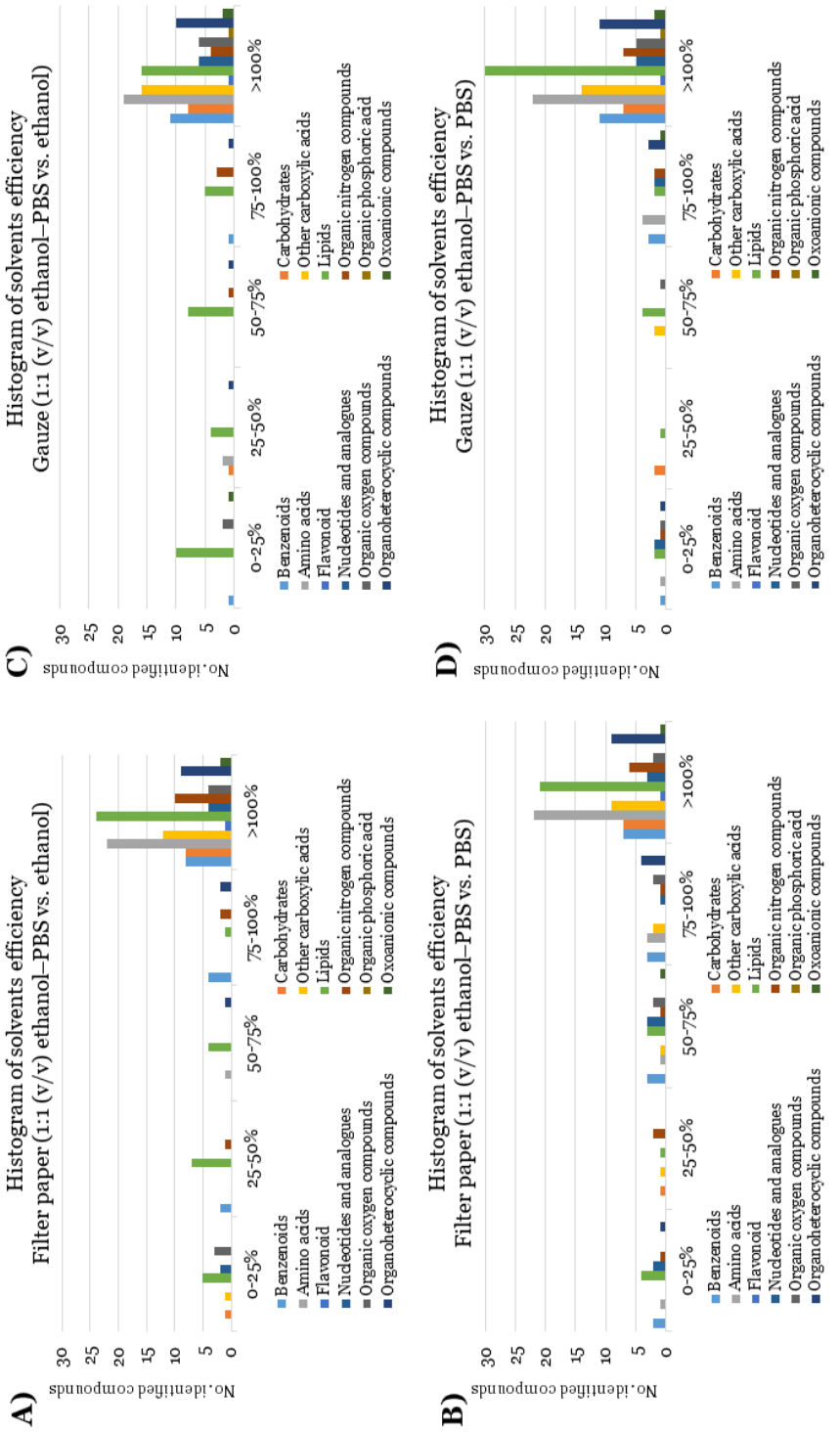

Fig. 2. Histograms representing the number of compounds tentatively identified in dry sweat collected on filter paper impregnated with 1:1 (v/v) ethanol–PBS. The reference (quantitative response set as 100%) corresponds to compounds identified in ethanol **(A)** and PBS **(B)**. Histograms representing the number of compounds tentatively identified in dry sweat collected by gauze impregnated with 1:1 (v/v) ethanol– PBS taking as 100% those identified with ethanol **(C)** and PBS **(D)**.

Complementarily, Fig. 2 (C and D) shows the bars diagrams with the results of the detection capability for gauze. Again, most compounds were more properly detected by using the ethanol–PBS solution. In this case, 20% of the metabolites identified when ethanol–PBS was used as solvent —mainly lipids such as fatty alcohols, long-chain fatty acids, sphingolipids and monoacylglycerols— were detected with higher efficiency when ethanol was used as solvent.

On the other hand, 17% of compounds (mainly benzenoids such as benzoic acid derivatives, proteinogenic amino acids and organoheterocyclic compounds such as indoles) were better detected with the buffer as compared to the ethanol–PBS mixture. According to these results, ethanol–PBS was the solvent providing the most complete coverage at qualitative and quantitative levels when this solution impregnated either gauze or filter paper. Further experiments were carried out with ethanol–PBS as suited solvent.

3.2. Comparison of the solid support for dry sweat sampling

The comparison between gauze and filter paper impregnated with ethanol–PBS was carried out by the Venn diagram illustrated in Fig. 3.

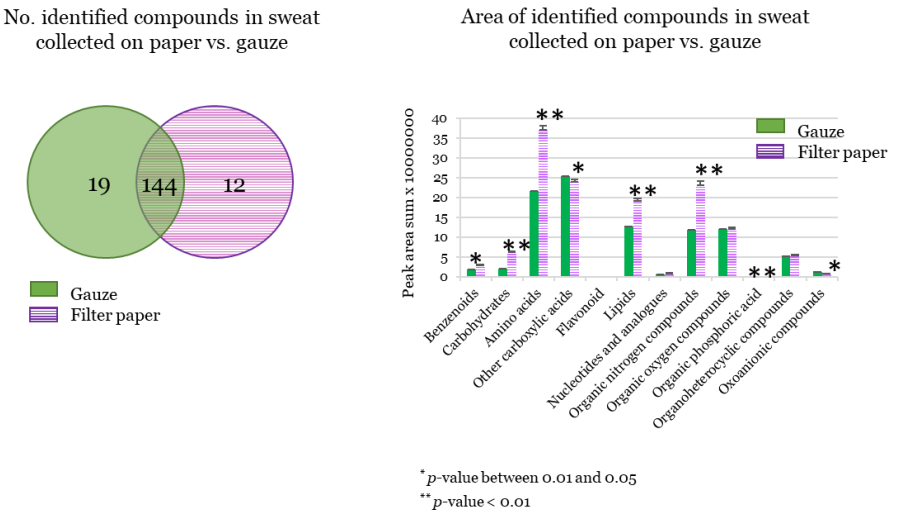

Fig. 3. Venn diagram and bar graph comparing the number and peak areas, respectively, of compounds tentatively identified in dry sweat collected on filter paper and gauze both impregnated with 1:1 (v/v) ethanol–PBS.

Nineteen metabolites were only identified by using gauze as support. They were particularly lipids —viz, fatty alcohols (C14:0 and C16:0) and medium-chain fatty acids (C6:0 and C8:0). On the other hand, 12 metabolites were only identified in impregnated filter paper. They included long-chain fatty acids —C20:0, C20:4 (n-6) and C18:2 (n-6)—, and monoacylglycerols (C12:0, C14:0 and C16:0) (Table 1).

Table 1. Compounds classified by chemical families, and tentatively identified by LC–QTOF MS/MS and GC–TOF/MS in dry sweat collected by gauze or filter paper impregnated with 1:1 (v/v) ethanol–PBS.

Identification	Compound name	RT (min)	Dry sweat (gauze)	Dry sweat (filter paper)
	Benzenoids			
LC—MS/MS	Anthranilic acid	1.91	✓	✓
LC—MS/MS	4-Hydroxybenzaldehyde	2.04	✓	✓
LC—MS/MS	2-Phenylacetamide	2.04	✓	✓
LC—MS/MS	3,4-Dihydroxybenzoic acid	4.95	✓	✓
LC—MS/MS	Hydroxyphenyllactic acid	5.14	✓	✓
LC—MS/MS	Chlorogenic acid	5.48	✓	✓
LC—MS/MS	Benzoic acid	5.82	✓	✓
LC—MS/MS	4-Acetoxyphenol	5.83	✓	✓
LC—MS/MS	Hydroquinone	6.01	✓	✓
GC—MS	ß-Ethenylbenzeneethanol	6.10	✓	✓
LC—MS/MS	3-Phenyllactic acid	6.58	✓	✓
LC—MS/MS	Phenylacetic acid	6.98	✓	✓
LC—MS/MS	Salicylic acid	7.83	✓	✓
GC—MS	Methylparaben	12.52	✓	✓
GC—MS	2,4,6-Trimethylbenzoic acid	12.54	✓	✓
GC—MS	Isovanillin	13.04	✓	✓
GC—MS	2-Phenyllactic acid	13.56	✓	✓
GC—MS	4-Methoxybenzoic acid	15.67		✓
GC—MS	Octocrylene	23.67	✓	✓
	Carbohydrates			
LC—MS/MS	Sorbitol	0.85	✓	✓
LC—MS/MS	Sucrose	0.92	✓	✓
GC—MS	Glyceric acid	10.26	✓	
GC—MS	Erythritol	12.56	✓	✓

Continuation Table 1

GC–MS	Threonic acid	13.00	✓	✓
GC–MS	Galactose	17.00	✓	✓
GC–MS	Glucose	17.04	✓	
GC–MS	Mannitol	17.33	✓	✓
GC–MS	Gluconic acid	17.97	✓	✓
GC–MS	Trehalose	24.18	✓	✓
	Amino acids			
LC–MS/MS	Arginine	0.85	✓	✓
LC–MS/MS	Citrulline	0.86	✓	✓
LC–MS/MS	Betaine	0.87	✓	✓
LC–MS/MS	N-Acetylhistidine	0.92	✓	✓
LC–MS/MS	1-Aminocyclopropane-1-carboxylic acid	1.36	✓	✓
LC–MS/MS	Isoleucine	1.59	✓	✓
LC–MS/MS	Tyrosine	2.04	✓	✓
LC–MS/MS	Phenylalanine	3.67	✓	✓
LC–MS/MS	Tryptophan	4.83	✓	✓
LC–MS/MS	N-Acetylvaline	4.98	✓	✓
GC–MS	Alanine	6.97	✓	✓
LC–MS/MS	Methyl N-(α-methylbutyryl)glycine	7.13	✓	✓
GC–MS	2-Aminobutyric acid	8.03	✓	✓
GC–MS	Valine	8.66	✓	✓
GC–MS	Proline	9.86	✓	✓
GC–MS	Glycine	9.96	✓	✓
GC–MS	Serine	10.68	✓	✓
GC–MS	Threonine	11.02	✓	✓
GC–MS	Sarcosine	11.25	✓	✓
GC–MS	Hydroxyproline	11.98	✓	✓
GC–MS	Aminomalonic acid	12.13	✓	✓
GC–MS	Pyroglutamic acid	12.80	✓	✓
GC–MS	α-Aminoadipic acid	13.10	✓	✓
GC–MS	2-Aminoheptanedioic acid	13.74	✓	✓
GC–MS	Ornithine	13.91	✓	✓
GC–MS	Glutamic acid	13.96	✓	✓
GC–MS	Asparagine	14.53	✓	✓
GC–MS	Histidine	17.22	✓	✓
GC–MS	Lysine	17.24	✓	✓

Continuation Table 1

	Carboxylic acids and derivatives			
LC—MS/MS	Citric acid	1.51	✓	✓
LC—MS/MS	2,2-Dimethylsuccinic acid	4.86	✓	✓
GC—MS	Lactic acid	6.29	✓	✓
GC—MS	Pyruvic acid	6.73	✓	✓
GC—MS	2-Hydroxybutyric acid	7.32	✓	✓
GC—MS	Oxalic acid	7.43	✓	✓
GC—MS	3-Hydroxypropionic acid	7.62	✓	✓
GC—MS	3-Hydroxybutyric acid	7.85	✓	✓
GC—MS	Carbamate	8.43	✓	✓
GC—MS	Hydroxycarbamic acid	9.02	✓	
GC—MS	Succinic acid	10.09	✓	
GC—MS	Fumaric acid	10.59	✓	✓
GC—MS	3,4-Dihydroxybutanoic acid	11.63	✓	
GC—MS	Malic acid	12.35	✓	✓
GC—MS	2-Oxoglutaric acid	13.43	✓	✓
GC—MS	Aconitic acid	15.42	✓	✓
GC—MS	Tributyl acetylcitrate	20.35	✓	✓
	Flavonoid			
LC—MS/MS	Rutin	6.22	✓	✓
	Lipids			
LC—MS/MS	2-Hexyldecanoic acid	0.89	✓	✓
LC—MS/MS	Stearic acid	0.89	✓	✓
LC—MS/MS	Propionylcarnitine	1.88	✓	✓
LC—MS/MS	Dexpanthenol	4.30	✓	✓
LC—MS/MS	2-Hydroxyisovaleric acid	4.61	✓	✓
LC—MS/MS	2-Methylbutyroylcarnitine	4.99	✓	✓
GC—MS	Caproic acid	6.55	✓	
GC—MS	3-Nonen-1-ol	7.14	✓	✓
LC—MS/MS	3-Hydroxycaprylic acid	7.63	✓	✓
LC—MS/MS	Hexadecasphinganine	8.08	✓	✓
LC—MS/MS	3-Ketosphingosine	8.23	✓	✓
LC—MS/MS	Phytosphingosine	8.76	✓	✓
LC—MS/MS	Sphinganine	8.82	✓	✓
GC—MS	2-Hydroxyisocaproic acid	8.92		✓
LC—MS/MS	Sphingosine	9.04	✓	✓

Continuation Table 1

LC–MS/MS	C75	9.15	✓	✓
LC–MS/MS	Myristoylcarnitine	9.32	✓	✓
GC–MS	Caprylic acid	9.41	✓	
LC–MS/MS	NN-Dimethylsphing-4-enine	9.81	✓	
LC–MS/MS	Palmitoylcarnitine	10.12	✓	✓
GC–MS	5-Hydroxy-N-valeric acid	10.34	✓	
LC–MS/MS	1-Palmitoyl-lysophosphatidylcholine (LysoPC(16:0))	11.34	✓	✓
GC–MS	1-Monoisobutyrylglycerol	12.13	✓	✓
GC–MS	Adipic acid	12.62	✓	
GC–MS	1-Dodecanol	13.40	✓	✓
GC–MS	Lauric acid	14.39	✓	✓
GC–MS	Tridecylic acid	15.49	✓	✓
GC–MS	1-Tetradecanol	15.64	✓	
GC–MS	Myristoleic acid	16.31	✓	✓
GC–MS	Myristic acid	16.54	✓	✓
GC–MS	Pentadecanoic acid	17.55	✓	✓
GC–MS	1-Hexadecanol	17.67	✓	
GC–MS	Hexadecanoic acid, ethyl ester	18.03	✓	✓
GC–MS	Palmitoleic acid	18.27	✓	✓
GC–MS	10-Heptadecenoic acid	19.19	✓	✓
GC–MS	Arachidonic acid	19.89		✓
GC–MS	Linoleic acid	19.90		✓
GC–MS	**1-Monolaurylglycerol**	19.92		✓
GC–MS	Oleic acid	20.08	✓	✓
GC–MS	1-**Monomyristylglycerol**	21.56		✓
GC–MS	Arachidic acid	21.97		✓
GC–MS	2-Monopalmitoylglycerol	22.83		✓
GC–MS	2-Monostearylglycerol	24.27	✓	✓
GC–MS	1-Monostearylglycerol	24.51	✓	✓
GC–MS	13-Docosenamide	24.59	✓	✓
GC–MS	Squalene	24.84	✓	✓
GC–MS	Cholesta-3,5-diene	25.55	✓	✓
GC–MS	Hexacosanoic acid	26.51	✓	✓
	Nucleosides, nucleotides, and analogues			
LC–MS/MS	Hypoxanthine	1.82	✓	✓

Continuation Table 1

LC–MS/MS	Inosine	3.64	✓	✓
LC–MS/MS	Guanosine	3.65	✓	✓
LC–MS/MS	Guanine	3.66	✓	✓
LC–MS/MS	Theophylline	4.95	✓	✓
LC–MS/MS	Caffeine	5.50	✓	✓
GC–MS	Thymine	11.24	✓	✓
GC–MS	Uric acid	18.94	✓	✓
	Organic nitrogen compounds			
LC–MS/MS	Spermine	0.75	✓	
LC–MS/MS	Choline	0.84	✓	✓
LC–MS/MS	Octylamine	4.70	✓	✓
GC–MS	2-Hydroxyethanamine	5.84		✓
LC–MS/MS	Aminopentol	8.17	✓	✓
LC–MS/MS	Hexaldecylamine	8.81	✓	✓
GC–MS	Urea	9.18	✓	✓
GC–MS	Ethanolamine	9.40	✓	✓
LC–MS/MS	Octadecylamine	9.62	✓	✓
LC–MS/MS	N,N'-Dicyclohexylurea	9.84	✓	✓
LC–MS/MS	Cetrimonium	10.27	✓	✓
GC–MS	Diethanolamine	11.71	✓	✓
GC–MS	1,3,5-Trihydroxy-tert-butylamine	12.62		✓
GC–MS	Triethanolamine	14.04	✓	✓
	Organic oxygen compounds			
GC–MS	2,3-Butanediol	5.85	✓	✓
GC–MS	1,2-Butanediol	8.08	✓	
GC–MS	4-Methyl-1,2-dihydroxypentane	8.83		✓
GC–MS	Diethylene glycol	9.08	✓	
GC–MS	Glycerol	9.48	✓	✓
GC–MS	2-(2-Butoxyethoxy)ethanol	10.43	✓	✓
GC–MS	1,1,1-Tris(hydroxyme-thyl)propane	11.12		✓
GC–MS	Pinitol	16.25	✓	✓
GC–MS	myo-Inositol	18.84	✓	✓
	Organic phosphoric acid			
GC–MS	Ethyl phosphoric acid	8.78	✓	
	Organoheterocyclic compounds			

Continuation Table 1

LC–MS/MS	Melamine	0.87	✓	✓
LC–MS/MS	Urocanic acid	1.34	✓	✓
LC–MS/MS	Nicotine	1.34	✓	✓
LC–MS/MS	Nicotinic acid	1.36	✓	✓
LC–MS/MS	Niacinamide	1.41	✓	✓
LC–MS/MS	2-Pyrimidine acetic acid	1.63	✓	✓
LC–MS/MS	Indole-3-acetamide	2.42	✓	✓
LC–MS/MS	4-Formyl indole	4.83	✓	✓
LC–MS/MS	3-Amino-2-naphthoic acid	5.87	✓	✓
GC–MS	Indane	6.10	✓	
LC–MS/MS	2-Methyl-3-hydroxy-ethylenepyran-4-one	7.64	✓	✓
LC–MS/MS	Embelin	10.04	✓	✓
GC–MS	3-Amino-2-piperidone	11.98	✓	✓
GC–MS	Alloxanoic acid	12.48	✓	✓
GC–MS	Cyanuric acid	13.79	✓	✓
GC–MS	Gluconic acid δ-lactone	16.83	✓	
	Oxoanionic compounds			
LC–MS/MS	Pyrophosphate	1.07	✓	✓
GC–MS	Phosphite (3:1)	7.06	✓	
GC–MS	Borate	9.68	✓	✓

It worth noting that the gauze used in this study was composed by hydrophilic healing material and a small quantity of nylon (polar tissue). Therefore, the gauze favored the dissolution of medium-chain fatty alcohols and fatty acids since the hydrophilic character of the polar head region of these compounds allowed setting interaction with the solid support. Concerning the filter paper, it was made of cotton linters pulp and high purity cellulose (above 95%) subjected to a rigorous washing with acids and water. Cellulose does not interact favorably with polar solvents such as water because its polymeric structure is based on strong intramolecular hydrogen bonds. Thus, filter paper favors dissolution of low polar compounds such as long-chain fatty acids and monoacylglycerols, characterized by an ester group and two alcohol groups, less polar than medium-chain fatty acids.

In addition to lipids, most families of compounds —*e.g.*, benzenoids (benzoic acid derivatives), carbohydrates (mainly sugar acids and sugar alcohols), hydroxy acids, amino acids, and organic nitrogen compounds (alkylamines and alkanolamines)— provided a

better response in the samples obtained using filter paper, except di- and tricarboxylic acids that were better detected in gauze, as shown in Fig. 3. Green bars are defined by the peak area of the compounds tentatively identified in dry sweat collected by gauze. Purple bars are defined by the peak area of the compounds identified in dry sweat collected by filter paper from the same six volunteers. In both cases, three replicates were performed. In fact, significant differences were found in lipids (p-value=0.000), amino acids (p-value=0.002), organic nitrogen compounds (p-value=0.002), carbohydrates (p-value=0.002), benzenoids (p-value=0.015) and other carboxylic acids (p-value=0.012) when the quantitative responses reported in dry sweat collected on the two supports were compared (Supplementary Table 1). Other families such as organic oxygen compounds (mainly diols) and organoheterocyclic compounds (a great diversity of compounds such as azines, indoles and pyridine derivatives, among others) behaved similarly in both supports. The methodological variability estimated as relative standard deviation of individual compounds ranged from 0.0 to 11.7% and from 0.0 to 17.7% for paper and gauze, respectively.

Supplementary Fig. 3 shows the BPCs from GC–MS and LC–MS obtained by analysis of dry sweat sampled on filter paper or gauze containing the same solvent mixture. Comparison of the BPCs shows that GC–MS, and LC–MS in negative ionization mode provided more and higher peaks at the beginning of the chromatogram, while LC–MS in positive ionization mode provided chromatograms with a higher number of peaks in overall terms along the chromatogram. Peaks identified at the beginning of the GC–MS chromatograms were mainly amino acids and hydroxy acids, while those identified in the middle part were sugars, other amino acids, long-chain fatty acids, alkanolamines and alcohols; the compounds identified at the end of the chromatograms were mainly lipids. LC–MS in negative ionization mode provided identification of compounds such as carbohydrates and fatty acyls at the beginning of the chromatogram. LC–MS in positive ionization presented peaks corresponding to compounds such as aromatic amino acids and nucleotides and analogues also stood out in the mid-part of the chromatogram, while lipids, mainly sphingolipids, acyl carnitines and alkylamines were identified at the end of the chromatogram (Supplementary Fig. 3). More polar compounds such as amino acids and organoheterocyclic compounds were tentatively identified at the first part of the chromatogram. Finally, it is worth mentioning the presence of one high-intensity peak detected by LC–MS in positive ionization of dry sweat collected with the gauze. This peak,

identified as an interferent (caprolactam), that also appears in gauze blanks. It is a key molecule in the synthesis of nylon.

3.3. Metabolome differences between dry and fresh sweat

Metabolomics analysis of fresh sweat had previously been addressed by three analytical platforms, namely, NMR, LC–MS/MS and GC–MS [4–6,25]. On the other hand, no comprehensive studies dealing with dry sweat metabolome composition had been carried out. As previously mentioned, most studies on dry sweat are referred to the collection of fresh sweat that was dried prior to analysis [7,8]. For this reason, a comparative study dealing with the analysis of dry and fresh sweat was carried out in this research by combined LC–MS/MS and GC–MS analyses. Supplementary Fig. 4 shows the BPCs obtained by analysis of both samples collected in similar body areas. Dry sweat reported more chromatographic peaks and with higher intensity than those detected in fresh sweat. However, the analysis of dry sweat led to the identification of 156 metabolites, while 172 were identified in fresh sweat.

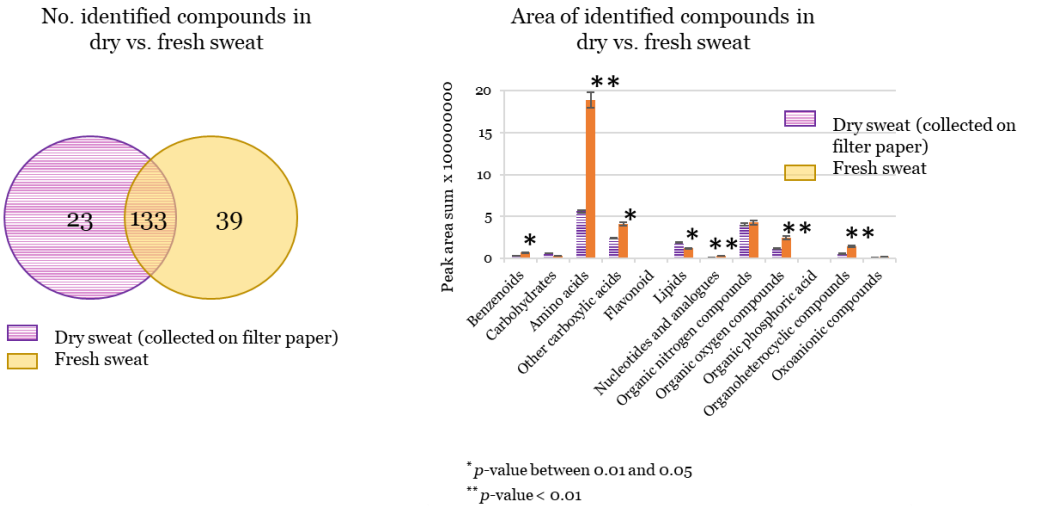

Fig. 4. Venn diagram and bar graph comparing the number and peak areas, respectively, of compounds tentatively identified in dry sweat collected on filter paper impregnated with 1:1 (v/v) ethanol–PBS and fresh sweat.

Fig. 4 shows that 133 metabolites were common to both samples, as listed in Table 2. Among the 23 compounds identified exclusively in dry sweat, 10 were lipids such as sphingolipids, monoacylglycerols and fatty acyls; 7 were benzenoids such as benzoic acid derivatives; and 4 were organic nitrogenated compounds such as alkylamines. It is well-known that when sweat is excreted to the skin surface, it interacts with the stratum corneum, the outermost layer of the epidermis consisting of dead cells (corneocytes). These cells are embedded in a lipid matrix of ceramides, cholesterol, and fatty acids [20].

Fresh sweat provided exclusively the tentative identification of polar compounds, mainly diols and carboxylic acids, and also of some no polar compounds, mainly fatty acids and derivatives.

Table 2. Compounds, classified by chemical families, tentatively identified by GC–TOF/MS and LC–QTOF MS/MS in dry sweat collected by filter paper impregnated with 1:1 (v/v) ethanol–PBS and in fresh sweat.

Identification	Compound name	RT (min)	Dry sweat (filter paper)	Fresh sweat
	Benzenoids			
LC—MS/MS	Anthranilic acid	1.91	✓	
LC—MS/MS	4-Hydroxybenzaldehyde	2.04	✓	✓
LC—MS/MS	2-Phenylacetamide	2.04	✓	✓
LC—MS/MS	3,4-Dihydroxybenzoic acid	4.95	✓	
LC—MS/MS	Hydroxyphenyllactic acid	5.14	✓	✓
LC—MS/MS	Chlorogenic Acid	5.48	✓	✓
LC—MS/MS	Benzoic acid	5.82	✓	✓
LC—MS/MS	4-Acetoxyphenol	5.83	✓	
LC—MS/MS	Hydroquinone	6.01	✓	
GC—MS	β-Ethenylbenzeneethanol	6.10	✓	✓
LC—MS/MS	3-Phenyllactic acid	6.58	✓	✓
LC—MS/MS	Phenylacetic acid	6.98	✓	✓
LC—MS/MS	Salicylic acid	7.83	✓	✓
LC—MS/MS	Ketoprofen	9.69		✓
GC—MS	Chlorphentermine	10.16		✓
GC—MS	3,4-Dimethylbenzoic acid	12.49		✓
GC—MS	Methylparaben	12.52	✓	✓
GC—MS	2,4,6-Trimethylbenzoic acid	12.54	✓	

Continuation Table 2

GC–MS	Isovanillin	13.04	✓	✓
GC–MS	2-Phenyllactic acid	13.56	✓	✓
GC–MS	4-Methoxybenzoic acid	15.67	✓	
GC–MS	Octocrylene	23.67	✓	
	Carbohydrates			
LC–MS/MS	Sorbitol	0.85	✓	✓
LC–MS/MS	Sucrose	0.92	✓	✓
GC–MS	Glyceric acid	10.26		✓
GC–MS	Erythritol	12.56	✓	✓
GC–MS	Threonic acid	13.00	✓	✓
GC–MS	Galactose	17.00	✓	✓
GC–MS	Glucose	17.04		✓
GC–MS	Mannitol	17.33	✓	✓
GC–MS	Gluconic acid	17.97	✓	✓
GC–MS	Trehalose	24.18	✓	✓
	Amino acids			
LC–MS/MS	Arginine	0.85	✓	✓
LC–MS/MS	Citrulline	0.86	✓	✓
LC–MS/MS	Betaine	0.87	✓	✓
LC–MS/MS	N-Acetylhistidine	0.92	✓	✓
LC–MS/MS	1-Aminocyclopropane-1-carboxylic acid	1.36	✓	✓
LC–MS/MS	Isoleucine	1.59	✓	✓
LC–MS/MS	Tyrosine	2.04	✓	✓
LC–MS/MS	Phenylalanine	3.67	✓	✓
LC–MS/MS	Tryptophan	4.83	✓	✓
LC–MS/MS	N-Acetylvaline	4.98	✓	✓
GC–MS	Alanine	6.97	✓	✓
LC–MS/MS	Methyl N-(α-methylbutyryl)glycine	7.13	✓	✓
GC–MS	2-Aminobutyric acid	8.03	✓	✓
GC–MS	Valine	8.66	✓	✓
GC–MS	Proline	9.86	✓	✓
GC–MS	Glycine	9.96	✓	✓
GC–MS	Serine	10.68	✓	✓
GC–MS	Threonine	11.02	✓	✓
GC–MS	Sarcosine	11.25	✓	✓
GC–MS	Hydroxyproline	11.98	✓	✓

Continuation Table 2

GC—MS	Aminomalonic acid	12.13	✓	✓
GC—MS	Aspartic acid	12.77		✓
GC—MS	Pyroglutamic acid	12.80	✓	✓
GC—MS	α-Aminoadipic acid	13.10	✓	✓
GC—MS	2-Aminoheptanedioic acid	13.74	✓	✓
GC—MS	Ornithine	13.91	✓	✓
GC—MS	Glutamic acid	13.96	✓	✓
GC—MS	Asparagine	14.53	✓	✓
GC—MS	Histidine	17.22	✓	✓
GC—MS	Lysine	17.24	✓	✓
	Carboxylic acids and derivatives			
LC—MS/MS	Citric acid	1.51	✓	✓
LC—MS/MS	2,2-Dimethylsuccinic acid	4.86	✓	✓
GC—MS	Lactic acid	6.29	✓	✓
GC—MS	Pyruvic acid	6.73	✓	✓
GC—MS	2-Hydroxybutyric acid	7.32	✓	✓
GC—MS	Oxalic acid	7.43	✓	✓
GC—MS	3-Hydroxypropionic acid	7.62	✓	✓
GC—MS	3-Hydroxybutyric acid	7.85	✓	✓
GC—MS	Carbamate	8.43	✓	✓
GC—MS	Hydroxycarbamic acid	9.02		✓
GC—MS	Succinic acid	10.09		✓
GC—MS	Fumaric acid	10.59	✓	✓
GC—MS	Glutaric acid	11.33		✓
GC—MS	3,4-Dihydroxybutanoic acid	11.63		✓
GC—MS	Malic acid	12.35	✓	✓
GC—MS	2-Oxoglutaric acid	13.43	✓	✓
GC—MS	Aconitic acid	15.42	✓	✓
GC—MS	Tributyl acetylcitrate	20.35	✓	✓
	Flavonoid			
LC—MS/MS	Rutin	6.22	✓	✓
	Lipids			
LC—MS/MS	Stearic acid	0.89	✓	✓
LC—MS/MS	2-Hexyldecanoic acid	0.89	✓	✓
LC—MS/MS	Propionylcarnitine	1.88	✓	✓
LC—MS/MS	Dexpanthenol	4.30	✓	✓

Continuation Table 2

LC–MS/MS	2-Hydroxyisovaleric acid	4.61	✓	✓
LC–MS/MS	2-Methylbutyroylcarnitine	4.99	✓	✓
GC–MS	Caproic acid	6.55		✓
GC–MS	3-Nonen-1-ol	7.14	✓	✓
LC–MS/MS	3-Hydroxycaprylic acid	7.63	✓	✓
LC–MS/MS	Hexadecasphinganine	8.08	✓	
LC–MS/MS	3-Ketosphingosine	8.23	✓	✓
GC–MS	3-Hydroxyisovaleric acid	8.59		✓
LC–MS/MS	Phytosphingosine	8.76	✓	✓
LC–MS/MS	Sphinganine	8.82	✓	
GC–MS	2-Hydroxyisocaproic acid	8.92	✓	✓
LC–MS/MS	Sphingosine	9.04	✓	✓
LC–MS/MS	C75	9.15	✓	✓
LC–MS/MS	Myristoylcarnitine	9.32	✓	
GC–MS	Caprylic acid	9.41		✓
LC–MS/MS	NN-Dimethylsphing-4-enine	9.81		✓
LC–MS/MS	Palmitoylcarnitine	10.12	✓	✓
GC–MS	5-Hydroxy-N-valeric acid	10.34		✓
GC–MS	Nonanoic acid	10.75		✓
LC–MS/MS	1-Palmitoyllysophos-phatidylcholine (LysoPC(16:0))	11.34	✓	
GC–MS	Capric acid	12.03		✓
GC–MS	1-Monoisobutyrylglycerol	12.13	✓	✓
GC–MS	Adipic acid	12.62		✓
GC–MS	1-Dodecanol	13.40	✓	✓
GC–MS	Lauric acid	14.39	✓	✓
GC–MS	Tridecylic acid	15.49	✓	✓
GC–MS	1-Tetradecanol	15.64		✓
GC–MS	Azelaic acid	15.97		✓
GC–MS	Myristoleic acid	16.31	✓	✓
GC–MS	Myristic acid	16.54	✓	✓
GC–MS	Pentadecanoic acid	17.55	✓	✓
GC–MS	1-Hexadecanol	17.67		✓
GC–MS	Hexadecanoic acid, ethyl ester	18.03	✓	
GC–MS	Palmitoleic acid	18.27	✓	✓
GC–MS	Palmitic acid	18.51		✓
GC–MS	10-Heptadecenoic acid	19.19	✓	✓

Continuation Table 2

GC—MS	Margaric acid	19.43		✓
GC—MS	1-Octadecanol	19.53		✓
GC—MS	Arachidonic acid	19.89	✓	
GC—MS	Linoleic acid	19.90	✓	
GC—MS	1-Monolaurylglycerol	19.92	✓	
GC—MS	Oleic acid	20.08	✓	✓
GC—MS	1-Monomyristylglycerol	21.56	✓	✓
GC—MS	Dehydroabietic acid	21.62		✓
GC—MS	Oleamide	21.76		✓
GC—MS	Arachidic acid	21.97	✓	✓
GC—MS	2-Monopalmitoylglycerol	22.83	✓	
GC—MS	1-Monopalmitoylglycerol	23.09		✓
GC—MS	Docosanoic acid	23.52		✓
GC—MS	2-Monostearylglycerol	24.27	✓	✓
GC—MS	1-Monostearylglycerol	24.51	✓	✓
GC—MS	13-Docosenamide	24.59	✓	✓
GC—MS	Squalene	24.84	✓	✓
GC—MS	Cholesta-3,5-diene	25.55	✓	
GC—MS	Hexacosanoic acid	26.51	✓	✓
	Nucleosides, nucleotides, and analogues			
LC—MS/MS	Hypoxanthine	1.82	✓	✓
LC—MS/MS	Inosine	3.64	✓	✓
LC—MS/MS	Guanosine	3.65	✓	✓
LC—MS/MS	Guanine	3.66	✓	✓
LC—MS/MS	Guanidylic acid	4.06		✓
LC—MS/MS	Theophylline	4.95	✓	✓
LC—MS/MS	Caffeine	5.50	✓	✓
GC—MS	Thymine	11.24	✓	✓
GC—MS	Uric acid	18.94	✓	✓
	Organic nitrogen compounds			
LC—MS/MS	Choline	0.84	✓	✓
LC—MS/MS	Octylamine	4.70	✓	✓
GC—MS	2-Hydroxyethanamine	5.84	✓	✓
LC—MS/MS	Aminopentol	8.17	✓	
LC—MS/MS	Hexaldecylamine	8.81	✓	
GC—MS	Urea	9.18	✓	✓

Continuation Table 2

GC—MS	Ethanolamine	9.40	✓	✓
LC—MS/MS	Octadecylamine	9.62	✓	
LC—MS/MS	N,N'-Dicyclohexylurea	9.84	✓	✓
LC—MS/MS	Cetrimonium	10.27	✓	
GC—MS	Diethanolamine	11.71	✓	✓
GC—MS	1,3,5-Trihydroxy-tert-butylamine	12.62	✓	✓
GC—MS	Triethanolamine	14.04	✓	✓
	Organic oxygen compounds			
GC—MS	2,3-Butanediol	5.85	✓	✓
GC—MS	Acetonyldimethylcarbinol	6.02		✓
GC—MS	2-Methyl-1,3-propanediol	6.63		✓
GC—MS	1,3-Butanediol	6.64		✓
GC—MS	Pinacol	7.78		✓
GC—MS	4-Methyl-1,2-dihydroxypentane	8.83	✓	
GC—MS	Diethylene glycol	9.08		✓
GC—MS	Glycerol	9.48	✓	✓
GC—MS	2-(2-Butoxyethoxy)ethanol	10.43	✓	
GC—MS	1,1,1-Tris(hydroxy-methyl)propane	11.12	✓	✓
GC—MS	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	13.31		✓
GC—MS	Pinitol	16.25	✓	✓
GC—MS	myo-Inositol	18.84	✓	✓
	Organic phosphoric acid			
GC—MS	Ethyl phosphoric acid	8.78		✓
	Organoheterocyclic compounds			
LC—MS/MS	Melamine	0.87	✓	✓
LC—MS/MS	Urocanic acid	1.34	✓	✓
LC—MS/MS	Nicotine	1.34	✓	✓
LC—MS/MS	Nicotinic acid	1.36	✓	✓
LC—MS/MS	Niacinamide	1.41	✓	✓
LC—MS/MS	2-Pyrimidine acetic acid	1.63	✓	✓
LC—MS/MS	Indole-3-acetamide	2.42	✓	✓
LC—MS/MS	4-Formyl indole	4.83	✓	✓
LC—MS/MS	3-Amino-2-naphthoic acid	5.87	✓	✓
GC—MS	Indane	6.10		✓
GC—MS	2-Furoic acid	7.50		✓

Continuation Table 2

LC–MS/MS	2-Methyl-3-hydroxy ethylenepyran-4-one	7.64	✓	✓
LC–MS/MS	Embelin	10.04	✓	✓
GC–MS	3-Amino-2-piperidone	11.98	✓	✓
GC–MS	Alloxanoic acid	12.48	✓	✓
GC–MS	Cyanuric acid	13.79	✓	✓
GC–MS	Gluconic acid δ-lactone	16.83		✓
	Oxoanionic compounds			
LC–MS/MS	Pyrophosphate	1.07	✓	✓
GC–MS	Borate	9.68	✓	✓

The comparison of the detection coverage provided by both samples allowed concluding that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds (Fig. 4). In fact, significant differences were found in the case of amino acids (p-value=0.000), nucleotides and analogues (p-value=0.002), organic oxygen compounds (alcohols) (p-value=0.000) and organoheterocyclic compounds (a diverse group constituted mainly by azines, indoles and pyridine derivatives) (p-value=0.001), but also in the case of benzenoids (benzaldehydes, benzoic acid derivatives and phenylpropanoic acids) (p-value=0.032), carboxylic acids (p-value=0.027) and lipids (p-value=0.031) in the comparison between fresh and dry sweat (Supplementary Table 2). Furthermore, the methodological variability, estimated as relative standard deviation of individual compounds, ranged from 0.0 to 11.7% and from 0.1 to 26.9% for dry and fresh sweat, respectively, which means that the protocol for collection of fresh sweat is less reproducible than that for sampling dry sweat. The solid support and the impregnation solvent play a key role favoring the dissolution of particular families of metabolites. It is remarkable that fresh sweat contained 85% of the metabolites detected in dry sweat collected by filter paper.

3.4. GC–MS vs LC–MS/MS for analysis of dry sweat

The combination of the two platforms allowed identification of 156 metabolites in dry sweat, with a detection coverage above 45 and 60% for LC–MS/MS and GC–MS, respectively.

Fig. 5 plots the Venn diagram that compares metabolites identified in dry sweat using both platforms. GC–MS resulted to be specially suited to detect lipids, and high polar compounds such as carbohydrates, carboxylic acids and amino acids. This approach allowed exclusively detecting organic oxygen compounds such as the polyols detected in dry sweat (Fig. 5). Bars are defined by the number of compounds tentatively identified in dry sweat from the six volunteers by GC–MS (purple) and LC–MS/MS (orange).

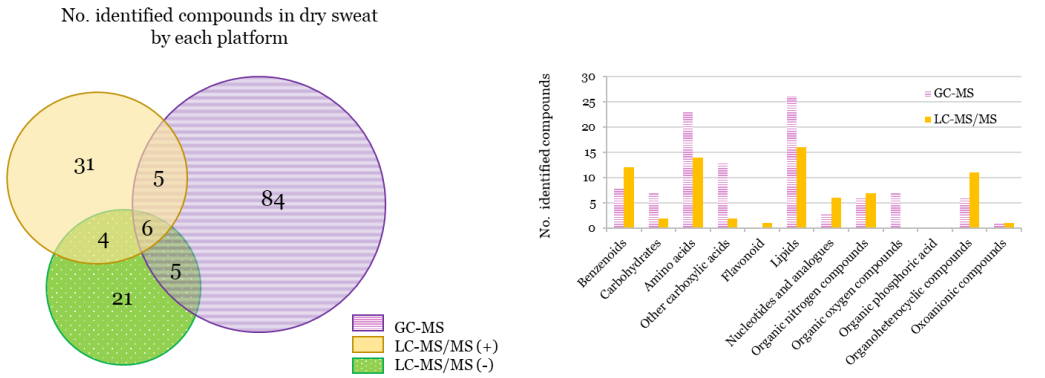

Fig. 5. Venn diagram comparing the number of compounds tentatively identified by GC–TOF/MS and LC–QTOF MS/MS (in both ionization modes) in dry sweat collected on filter paper impregnated with 1:1 (v/v) ethanol–PBS, classified by families in the bar graph.

On the other hand, LC–MS/MS was capable of covering a higher number of amino acids, nucleotides and analogues, organic nitrogen compounds such as alkylamines and alkanolamines, benzenoids (mainly benzoic acid derivatives), and organoheterocyclic compounds (mainly pyridine derivatives). These families were easily identified in positive ionization mode, except benzenoids, which reported higher sensitivity when working in negative mode. In fact, comparing the relative abundance (%) of the families identified in dry sweat by LC–MS/MS in positive and negative ionization modes, it is remarkable that the former mode was the best as it covers a high percentage of polar lipids (such as carnitines and sphingolipids), organic nitrogen compounds (such as monoalkylamines) and organoheterocyclic compounds (such as pyridine derivatives). The positive ionization was also useful to detect xanthines and amino acids in sweat, while the negative polarity was better for covering nucleotides, carbohydrates, some carboxylic acids and benzenoids

such as benzoic acids, and also for detecting hydroxy fatty acids in dry sweat (Supplementary Fig. 5).

Supplementary Table 3 lists the tentatively identified metabolites in dry sweat by LC–MS, classified according to their chemical family. Among the identified lipids, it is remarkable the presence of sphingosines, acylcarnitines and a glycerophospholipid. Regarding benzenoids and organoheterocyclic metabolites, pyridine derivatives (niacinamide and nicotinic acid), indoles, azines and other organoheterocyclic compounds (embelin, 3-amino-2-naphthoic acid and 2-methyl-3-hydroxyethylenepyran-4-one), and benzenoids such as benzoic acid derivatives and phenylpropanoic acids were identified for the first time in sweat. Moreover, alkylamines, aminopentol and N,N'-dicyclohexylurea detected in dry sweat had not been previously identified. Other metabolites detected in dry sweat were sugars, nucleotides, amino acids, N-acyl amino acids (methyl N-(a-methylbutyryl)glycine, N-acetylhistidine and N-acetylvaline), 2,2-dimethyl succinic acid, rutin, guanosine, guanine, hypoxanthine and pyrophosphate. Although N-acetylamino acids had not been previously identified in sweat, similar metabolites as N-acetyl-aspartate and N-acetyl-serine had been identified in fresh sweat by NMR and MS, respectively [4,26].

Complementarily, Supplementary Table 4 lists the tentatively identified metabolites in dry sweat by GC–MS, classified according to their chemical family. Some lipids such as 1-monoisobutyrylglycerol, 1-monolaurylglycerol, hexadecanoic acid ethyl ester and 13-docosenamide identified in dry sweat had not been previously identified in fresh sweat. Also, polar compounds such as amino acids, carboxylic acids (aconitic acid and 2-oxoglutaric acid), hydroxy acids (3-hydroxybutyric and 3-hydroxypropionic acids), sugars (trehalose, gluconic acid, erythritol and mannitol) were identified in dry sweat. These compounds, but amino acids, had not been previously identified in fresh sweat [23,27,28]. Other metabolites detected in dry sweat for the first time were benzenoids (such as isovanillin, 2,4,6-trimethylbenzoic acid, 4-methoxybenzoic acid, phenyllactic acid, ß-ethenylbenzeneethanol and octorylene), alcohols (pinitol and 4-methyl-1,2-dihydroxypentane), organoheterocyclic compounds (cyanuric acid, melamine and alloxanoic acid), alkanolamines (2-hydroxyethanamine and 1,3,5-trihydroxy-tert-butylamine), and uric acid and thymine.

4. Conclusions and perspectives

Sampling approaches for collection of dry sweat have been evaluated to propose a standardized protocol alternative to fresh sweat sampling. The use of paper impregnated with 1:1 (v/v) ethanol–PBS led to the best results in terms of detection coverage by using a combined approach based on GC–MS and LC–MS/MS analysis. A total 175 compounds were tentatively identified in dry sweat. The comparison between dry and fresh sweat revealed that the former provided an improvement in the detection of low-polar compounds, while fresh sweat was more suited for detection of polar metabolites. Nevertheless, particular families such as carnitines, sphingolipids and N-acyl-amino acids of essential amino acids were detected in dry sweat and have not been reported in fresh sweat. Considering the variability sources affecting the sampling of fresh sweat, collection of dry sweat can be proposed as a standardized approach to use this biofluid in metabolomics studies.

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Supplementary material

Supplementary Table 1. Kruskal-Wallis statistical analysis to evaluate significant differences in the dissolution levels of the tentatively identified compounds (classified by chemical families) in dry sweat collected by filter paper or gauze impregnated with 1:1 (v/v) ethanol–PBS.

Family	*p*-Value[a]
Benzenoids	0.015*
Carbohydrates	0.002**
Amino acids	0.002**
Other carboxylic acids	0.012*
Flavonoid	0.905
Lipids	0.000**
Nucleotides and analogues	0.153
Organic nitrogen compounds	0.002**
Organic oxygen compounds	0.280
Organic phosphoric acid	0.000**
Organoheterocyclic compounds	0.410
Oxoanionic compounds	0.011*

[a] *p*-Values were obtained by Kruskal-Wallis test comparing areas of the compounds in dry sweat collected on gauze or paper impregnated with the solvent mixture; * *p*-Value between 0.01 and 0.05; ** *p*-Value < 0.01.

Supplementary Table 2. Kruskal-Wallis statistical analysis to evaluate the significance differences in the levels of the tentatively identified compounds (classified by chemical families) in dry sweat collected by filter paper impregnated with 1:1 (v/v) ethanol–PBS and in fresh sweat.

Family	*p*-Value[a]
Benzenoids	0.032*
Carbohydrates	0.270
Amino acids	0.000**
Other carboxylic acids	0.027*
Flavonoid	0.711
Lipids	0.031*
Nucleotides and analogues	0.002**
Organic nitrogen compounds	0.131
Organic oxygen compounds	0.000**
Organoheterocyclic compounds	0.001**
Oxoanionic compounds	0.712

[a] *p*-Values were obtained by Kruskal-Wallis test comparing areas of the compounds in dry sweat collected by paper impregnated with the solvent mixture and in fresh sweat; * *p*-value between 0.01 and 0.05; ** *p*-value < 0.01.

Supplementary Table 3. Compounds, classified by chemical families, tentatively identified by LC–QTOF MS/MS in dry sweat collected by filter paper impregnated with 1:1 (v/v) ethanol–PBS (pH 7).

Compound name	RT (min)	Experimental MS spectrum (*m/z*)	Theoretical MS spectrum (*m/z*)	Error (ppm)	Adduct	Product ions (*m/z*)	Family	Subfamily
4-Hydroxybenzaldehyde	2.04	123.0441	123.0441	0.0	$[M+H]^+$	51.0221 77.0377 95.0481	Benzenoids	Benzaldehyde
Hydroquinone	6.01	109.0296	109.0295	0.9	$[M-H]^-$	108.0217	Benzenoids	Benzenediol
Anthranilic acid	1.91	136.0405	136.0404	0.7	$[M-H]^-$	65.0391 92.0497	Benzenoids	Benzoic acids and derivatives
Benzoic acid	5.82	121.0290	121.0295	-4.1	$[M-H]^-$	77.0389	Benzenoids	Benzoic acids and derivatives
3,4-Dihydroxybenzoic acid	4.95	153.0192	153.0193	-0.7	$[M-H]^-$	91.0279 108.0218 109.0273	Benzenoids	Benzoic acids and derivatives
Salicylic acid	7.83	137.0247	137.0244	2.2	$[M-H]^-$	65.0381 93.0345	Benzenoids	Benzoic acids and derivatives
4-Acetoxyphenol	5.83	151.0404	151.0401	2.0	$[M-H]^-$	108.0218	Benzenoids	Phenol derivative
2-Phenylacetamide	2.04	136.0752	136.0757	-3.7	$[M+H]^+$	91.054 119.0483	Benzenoids	Phenylacetamide
Hydroxyphenyllactic acid	5.14	181.0506	181.0506	0.0	$[M-H]^-$	119.05 135.0444 163.0387	Benzenoids	Phenylpropanoic acids
3-Phenyllactic acid	6.58	165.0564	165.0557	4.2	$[M-H]^-$	72.9921 103.0548 119.0488	Benzenoids	Phenylpropanoic acids
Chlorogenic acid	5.48	353.0878	353.0878	0.0	$[M-H]^-$	179.0342 191.0555	Benzenoids	Others
Phenylacetic acid	6.98	137.0592	137.0597	-3.6	$[M+H]^+$	65.0387 91.0541 119.047	Benzenoids	Others
Sucrose	0.92	341.1108	341.1089	5.6	$[M-H]^-$	59.0142 89.0246 341.111	Carbohydrates	Disaccharide
Sorbitol	0.85	181.0719	181.0718	0.6	$[M-H]^-$	59.0142 71.0140 89.0238	Carbohydrates	Sugar alcohol
Arginine	0.85	175.1189	175.1190	-0.6	$[M+H]^+$	60.0547 70.0645 116.0707	Amino acids	α-Amino acids

Continuation Supplementary Table 3

Compound name	RT (min)	Experimental MS spectrum (*m/z*)	Theoretical MS spectrum (*m/z*)	Error (ppm)	Adduct	Product ions (*m/z*)	Family	Subfamily
Citrulline	0.86	176.1026	176.1030	-2.3	$[M+H]^+$	70.0645 113.0705 159.0745	Amino acids	α-Amino acids
Isoleucine	1.59	132.1019	132.1019	0.0	$[M+H]^+$	57.0571 69.0697 86.0958	Amino acids	α-Amino acids
Tyrosine	2.04	182.0804	182.0812	-4.4	$[M+H]^+$	91.0544 123.0444 136.0749	Amino acids	α-Amino acids
Phenylalanine	3.67	166.0862	166.0863	-0.6	$[M+H]^+$	93.0688 103.0541 120.0803	Amino acids	α-Amino acids
Tryptophan	4.83	205.0972	205.0972	0.0	$[M+H]^+$	118.0645 146.0594 159.0913	Amino acids	α-Amino acids
Betaine	0.87	118.0863	118.0863	0.0	$[M]^+$	58.0645 59.0726	Amino acids	α-Amino acid derivatives
1-Aminocyclopropane-1-carboxylic acid	1.36	102.0553	102.0555	-2.0	$[M+H]^+$	56.0493	Amino acids	α-Amino acid derivatives
N-Acetylhistidine	0.92	198.0869	198.0873	-2.0	$[M+H]^+$	95.0594 156.0758 110.0708	Amino acid derivatives	N-acyl-α-amino acids
N-Acetylvaline	4.98	158.0824	158.0823	0.6	$[M-H]^-$	114.0907 116.0713	Amino acid derivatives	N-acyl-α-amino acids
Methyl N-(α-methylbutyryl)glycine	7.13	187.0977	187.0976	0.5	$[M-H]^-$	57.0344 97.0657 125.0967	Amino acid derivatives	N-acyl-α-amino acids
2,2-Dimethylsuccinic acid	4.86	145.0506	145.0506	0.0	$[M-H]^-$	83.0481 101.0609	Carboxylic acids and derivatives	Dicarboxylic acid derivative
Citric acid	1.51	191.0193	191.0197	-2.1	$[M-H]^-$	67.0180 87.0086 111.0084	Carboxylic acids and derivatives	Tricarboxylic acid
Rutin	6.22	609.1452	609.1461	-1.5	$[M-H]^-$	301.0326	Flavonoid	Flavonoid-3-O-glycoside
Propionylcarnitine	1.88	218.1379	218.1387	-3.7	$[M]^+$	56.9867 60.0809 85.0281	Lipids	Acyl carnitines
2-Methylbutyroylcarnitine	4.99	246.1701	246.1700	0.4	$[M]^+$	60.0791 85.0276 86.0318	Lipids	Acyl carnitines
Myristoylcarnitine	9.32	372.3105	372.3108	-0.8	$[M]^+$	60.0798 85.0278 313.2353	Lipids	Acyl carnitines

Continuation Supplementary Table 3

Compound name	RT (min)	Experimental MS spectrum (*m/z*)	Theoretical MS spectrum (*m/z*)	Error (ppm)	Adduct	Product ions (*m/z*)	Family	Subfamily
Palmitoylcarnitine	10.12	400.3403	400.3421	-4.5	$[M]^+$	85.0275 341.2637	Lipids	Acyl carnitines
2-Hydroxyisovaleric acid	4.61	117.0549	117.0557	-6.8	$[M-H]^-$	49.0343 71.0495	Lipids	Hydroxy fatty acids
3-Hydroxycaprylic acid	7.63	159.1026	159.1027	-0.6	$[M-H]^-$	59.0141	Lipids	Hydroxy fatty acids
C75	9.15	253.1447	253.1445	0.8	$[M-H]^-$	209.1535 253.1435	Lipids	Inhibitor
2-Hexyldecanoic acid	0.89	255.2330	255.2330	0.0	$[M-H]^-$		Lipids	Long-chain fatty acids
Stearic acid	0.89	283.2641	283.2643	-0.7	$[M-H]^-$		Lipids	Long-chain fatty acids
Dexpanthenol	4.3	206.1386	206.1387	-0.5	$[M+H]^+$	58.0647 76.0753 188.1278	Lipids	N-acyl amine
1-Palmitoyllysophos-phatidylcholine (LysoPC(16:0))	11.34	496.3370	496.3398	-5.6	$[M]^+$	60.0801 104.1059 184.0735	Lipids	1-Acyl-sn-glycero-3-phosphocholine
Hexadecasphinganine	8.08	274.2742	274.2741	0.4	$[M+H]^+$	88.0757 102.0913 256.2635	Lipids	1,2-Aminoalcohols
3-Ketosphingosine	8.23	298.2744	298.2741	1.0	$[M+H]^+$	60.0444 72.0444 280.2635	Lipids	1,2-Aminoalcohols
Sphinganine	8.82	302.3058	302.3054	1.3	$[M+H]^+$	102.0913 106.0863 284.2948	Lipids	1,2-Aminoalcohols
Sphingosine	9.04	300.2891	300.2897	-2.0	$[M+H]^+$	252.2668 264.2665 282.2786	Lipids	1,2-Aminoalcohols
Phytosphingosine	8.76	318.2994	318.3003	-2.8	$[M+H]^+$	60.0441 282.2781 300.2883	Lipids	1,3-Aminoalcohol
Theophylline	4.95	181.0716	181.0720	-2.2	$[M+H]^+$	69.0447 96.0549 124.0499	Nucleosides, nucleotides, and analogues	Xanthines
Inosine	3.64	267.0735	267.0735	0.0	$[M-H]^-$	135.0309	Nucleosides, nucleotides, and analogues	Purine nucleosides
Guanosine	3.65	282.0844	282.0844	0.0	$[M-H]^-$	108.7162 133.0156 150.0426	Nucleosides, nucleotides, and analogues	Purine nucleosides
Hypoxanthine	1.82	137.0451	137.0458	-5.1	$[M+H]^+$	55.0293 82.0383 94.0399	Nucleosides, nucleotides, and analogues	Hypoxanthine

Continuation Supplementary Table 3

Compound name	RT (min)	Experimental MS spectrum (*m/z*)	Theoretical MS spectrum (*m/z*)	Error (ppm)	Adduct	Product ions (*m/z*)	Family	Subfamily
Guanine	3.66	152.0565	152.0567	-1.3	$[M+H]^+$	110.0339 135.0292	Nucleosides, nucleotides, and analogues	Purine derivatives
Caffeine	5.50	195.0878	195.0877	0.5	$[M+H]^+$	110.0694 123.0413 138.0664	Nucleosides, nucleotides, and analogues	Xanthines
Aminopentol	8.17	406.3528	406.3527	0.2	$[M+H]^+$	88.0757 132.1019 146.1176	Organic nitrogen compounds	1,2-Aminoalcohols
Choline	0.84	104.1069	104.1070	-1.0	$[M]^+$	45.0333 58.0645 60.0813	Organic nitrogen compounds	Cholines
Cetrimonium	10.27	284.3314	284.3312	0.7	$[M+H]^+$	57.0695 60.0804 71.0853	Organic nitrogen compounds	Tetraalkylamine
N,N'-Dicyclohexylurea	9.84	225.1958	225.1961	-1.3	$[M+H]^+$	61.0389 83.0856 143.1169	Organic nitrogen compounds	Urea
2-Pyrimidine acetic acid	1.63	137.0353	137.0357	-2.9	$[M-H]^-$	66.0350 93.0456	Organoheterocyclic compounds	1,3-Diazine
Urocanic acid	1.34	139.0494	139.0502	-5.8	$[M+H]^+$	66.0348 93.0448 121.0394	Organoheterocyclic compounds	Imidazolyl carboxylic acid
Indole-3-acetamide	2.42	175.0862	175.0866	-2.3	$[M+H]^+$	70.9407 130.0637 158.0600	Organoheterocyclic compounds	3-Alkylindole
4-Formyl índole	4.83	146.0596	146.0600	-2.7	$[M+H]^+$	91.0536 118.0633 119.0683	Organoheterocyclic compounds	Others
3-Amino-2-naphthoic acid	5.87	188.0698	188.0706	-4.3	$[M+H]^+$	115.0536 142.0646 170.0591	Organoheterocyclic compounds	Others
2-Methyl-3-hydroxyethylene-pyran-4-one	7.64	151.0400	151.0401	-0.7	$[M-H]^-$	92.0269 136.0161	Organoheterocyclic compounds	Others
Nicotinic acid	1.36	124.0386	124.0393	-5.6	$[M+H]^+$	78.0335 80.0486	Organoheterocyclic compounds	Pyridinecarboxylic acids
Niacinamide	1.41	123.0548	123.0553	-4.1	$[M+H]^+$	78.0335 80.0480	Organoheterocyclic compounds	Pyridinecarboxylic acids
Nicotine	1.34	163.1223	163.1230	-4.3	$[M+H]^+$	106.0645 117.0567 130.0644	Organoheterocyclic compounds	Pyrrolidinylpyridine
Embelin	10.04	293.1763	293.1758	1.7	$[M-H]^-$	96.9598 221.1542	Organoheterocyclic compounds	Others

Continuation Supplementary Table 3

Compound name	RT (min)	Experimental MS spectrum (*m/z*)	Theoretical MS spectrum (*m/z*)	Error (ppm)	Adduct	Product ions (*m/z*)	Family	Subfamily
Melamine	0.87	127.0718	127.0727	-7.1	$[M+H]^+$	68.0238 85.0499	Organohetero-cyclic compounds	1,3,5-Triazine
Octylamine	4.7	130.1587	130.1590	-2.3	$[M+H]^+$	44.0495 57.0699 74.0964	Organic nitrogen compounds	Monoalkylamines
Hexaldecylamine	8.81	242.2848	242.2842	2.5	$[M+H]^+$	57.0693 71.0841 242.2844	Organic nitrogen compounds	Monoalkylamines
Octadecylamine	9.62	270.3158	270.3155	1.1	$[M+H]^+$	57.0679	Organic nitrogen compounds	Monoalkylamines
Pyrophosphate	1.07	176.9356	176.9359	-1.7	$[M-H]^-$	78.9589 158.9262	Oxoanionic compound	Non-metal pyrophosphate

Supplementary Table 4. Compounds, classified by chemical families, tentatively identified by GC–TOF/MS in dry sweat collected by filter paper impregnated with 1:1 (v/v) ethanol–PBS (pH 7).

Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
Isovanillin	13.04	$C_8H_8O_3$	621-59-0	$C_{11}H_{16}O_3Si$	224.0865 209.0633 194.0398	Benzenoids	Benzaldehyde
Methylparaben	12.52	$C_8H_8O_3$	99-76-3	$C_{11}H_{16}O_3Si$	224.0863 209.0622 193.0666	Benzenoids	Benzoic acids and derivatives
2,4,6-Trimethyl-benzoic acid	12.54	$C_{10}H_{12}O_2$	480-63-7	$C_{13}H_{20}O_2Si$	221.0987 119.0848 91.0538	Benzenoids	Benzoic acids and derivatives
4-Methoxybenzoic acid	15.67	$C_8H_8O_3$	100-09-4	$C_{11}H_{16}O_3Si$	209.0630 165.0731 135.0444	Benzenoids	Benzoic acids and derivatives
β-Ethenylben-zeneethanol	6.10	$C_{10}H_{12}O$	6052-63-7	$C_{10}H_{12}O$	117.0691 91.0534 51.0118	Benzenoids	Others
Octocrylene	23.67	$C_{24}H_{27}NO_2$	6197-30-4	$C_{24}H_{27}NO_2$	360.1961 232.0762 178.0770	Benzenoids	Others
2-Phenyllactic acid	13.56	$C_9H_{10}O_3$	828-01-3	$C_{15}H_{26}O_3Si_2$	220.0909 193.1041 91.0537	Benzenoids	Phenylpropanoic acid
Trehalose	24.18	$C_{12}H_{22}O_{11}$	6138-23-4	$C_{36}H_{86}O_{11}Si_8$	361.1689 243.1224 191.0900	Carbohydrates	Disaccharide
Galactose	17.00	$C_6H_{12}O_6$	59-23-4	$C_{21}H_{52}O_6Si_5$	305.1384 265.1097 204.0998	Carbohydrates	Monosaccharide
Threonic acid	13.00	$C_4H_8O_5$	20246-26-8	$C_{16}H_{40}O_5Si_4$	292.1326 220.0931 205.1057	Carbohydrates	Sugar acids
Gluconic acid	17.97	$C_6H_{12}O_7$	526-95-4	$C_{24}H_{60}O_7Si_6$	333.1387 292.1350 117.0371	Carbohydrates	Sugar acids
Erythritol	12.56	$C_4H_{10}O_4$	149-32-6	$C_{16}H_{42}O_4Si_4$	217.1075 205.1066 117.0432	Carbohydrates	Sugar alcohols
Mannitol	17.33	$C_6H_{14}O_6$	69-65-8	$C_{24}H_{62}O_6Si_6$	319.1590 277.1472 217.1086	Carbohydrates	Sugar alcohols

Continuation Supplementary Table 4

Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
Alanine	6.97	$C_3H_7NO_2$	56-41-7	$C_9H_{23}NO_2Si_2$	218.1013 190.1067 116.0885	Amino acids	α-Amino acids
2-Aminobutyric acid	8.03	$C_4H_9NO_2$	2835-81-6	$C_{10}H_{25}NO_2Si_2$	204.1224 142.0692 130.1041	Amino acids	α-Amino acids
Valine	8.66	$C_5H_{11}NO_2$	72-18-4	$C_{11}H_{27}NO_2Si_2$	246.1324 218.1110 203.0809	Amino acids	α-Amino acids
Proline	9.86	$C_5H_9NO_2$	609-36-9	$C_{11}H_{25}NO_2Si_2$	216.1224 170.0620 142.1043	Amino acids	α-Amino acids
Glycine	9.96	$C_2H_5NO_2$	56-40-6	$C_{11}H_{29}NO_2Si_3$	276.1248 248.1303 174.1125	Amino acids	α-Amino acids
Serine	10.68	$C_3H_7NO_3$	302-84-1	$C_{12}H_{31}NO_3Si_3$	218.1033 204.1283 188.0924	Amino acids	α-Amino acids
Threonine	11.02	$C_4H_9NO_3$	72-19-5	$C_{13}H_{33}NO_3Si_3$	291.1513 218.1342 117.0734	Amino acids	α-Amino acids
Sarcosine	11.25	$C_3H_7NO_2$	107-97-1	$C_9H_{23}NO_2Si_2$	190.1074 147.0659 116.0888	Amino acids	α-Amino acids
Hydroxyproline	11.98	$C_5H_9NO_3$	51-35-4	$C_{14}H_{33}NO_3Si_3$	230.1212 214.0909 188.0928	Amino acids	α-Amino acids
Aminomalonic acid	12.13	$C_3H_5NO_4$	1068-84-4	$C_{12}H_{29}NO_4Si_3$	320.1163 292.1216 218.1028	Amino acids	α-Amino acids
Pyroglutamic acid	12.80	$C_5H_7NO_3$	149-87-1	$C_8H_{15}NO_3Si$	106.0635 84.0445 56.0497	Amino acids	α-Amino acids
α-Aminoadipic acid	13.10	$C_6H_{11}NO_4$	1118-90-7	$C_{15}H_{35}NO_4Si_3$	334.1635 260.1497 100.0218	Amino acids	α-Amino acids
2-Aminoheptanedioic acid	13.74	$C_7H_{13}NO_4$	627-76-9	$C_{16}H_{37}NO_4Si_3$	274.1660 218.1033 73.0470	Amino acids	α-Amino acids
Ornithine	13.91	$C_5H_{12}N_2O_2$	70-26-8	$C_{14}H_{36}N_2O_2Si_3$	348.2070 142.1039 115.0789	Amino acids	α-Amino acids
Glutamic acid	13.96	$C_5H_9NO_4$	56-86-0	$C_{14}H_{33}NO_4Si_3$	348.1465 246.1333 230.1019	Amino acids	α-Amino acids
Asparagine	14.53	$C_4H_8N_2O_3$	70-47-3	$C_{13}H_{32}N_2O_3Si_3$	231.1341 188.0919 116.0842	Amino acids	α-Amino acids

Continuation Supplementary Table 4

Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
Histidine	17.22	$C_6H_9N_3O_2$	71-00-1	$C_{15}H_{33}N_3O_2Si_3$	254.1495 218.1029 154.0918	Amino acids	α-Amino acids
Lysine	17.24	$C_6H_{14}N_2O_2$	657-27-2	$C_{18}H_{46}N_2O_2Si_4$	362.2214 230.1162 156.1187	Amino acids	α-Amino acids
Pyruvic acid	6.73	$C_3H_4O_3$	127-17-3	$C_7H_{15}NO_3Si$	189.0811 174.0583 158.0625	Carboxylic acids and derivatives	α-Keto acid
Carbamate	8.43	CH_3NO_2	302-11-4	$C_{10}H_{27}NO_2Si_3$	262.1110 174.0575 100.0222	Carboxylic acids and derivatives	Carbamic acid
Oxalic acid	7.43	$C_2H_2O_4$	144-62-7	$C_8H_{18}O_4Si_2$	219.0480 190.0831 131.0346	Carboxylic acids and derivatives	Dicarboxylic acids
Fumaric acid	10.59	$C_4H_4O_4$	110-17-8	$C_{10}H_{20}O_4Si_2$	245.0659 217.0707 143.0518	Carboxylic acids and derivatives	Dicarboxylic acids
Malic acid	12.35	$C_4H_6O_5$	617-48-1	$C_{13}H_{30}O_5Si_3$	245.0660 233.1022 217.0713	Carboxylic acids and derivatives	Dicarboxylic acids
2-Oxoglutaric acid	13.43	$C_5H_6O_5$	328-50-7	$C_{12}H_{25}NO_5Si_2$	304.1019 288.1085 198.0581	Carboxylic acids and derivatives	Dicarboxylic acids
Lactic acid	6.29	$C_3H_6O_3$	79-33-4	$C_9H_{22}O_3Si_2$	233.1064 219.0923 203.0604	Carboxylic acids and derivatives	Hydroxy acids
2-Hydroxybutyric acid	7.32	$C_4H_8O_3$	20016-85-7	$C_{10}H_{24}O_3Si_2$	233.1037 131.0886 66.0226	Carboxylic acids and derivatives	Hydroxy acids
3-Hydroxypropionic acid	7.62	$C_3H_6O_3$	503-66-2	$C_9H_{22}O_3Si_2$	219.0867 177.0749 66.0247	Carboxylic acids and derivatives	Hydroxy acids
3-Hydroxybutyric acid	7.85	$C_4H_8O_3$	300-85-6	$C_{10}H_{24}O_3Si_2$	233.1019 191.0915 117.0719	Carboxylic acids and derivatives	Hydroxy acids
Aconitic acid	15.42	$C_6H_6O_6$	4023-65-8	$C_{15}H_{30}O_6Si_3$	375.1119 229.1073 147.0658	Carboxylic acids and derivatives	Tricarboxylic acid derivative
Tributyl acetylcitrate	20.35	$C_{20}H_{34}O_8$	77-90-7	$C_{20}H_{34}O_8$	259.1535 185.0815 129.0213	Carboxylic acids and derivatives	Tricarboxylic acid
Hexadecanoic acid, ethyl ester	18.03	$C_{18}H_{36}O_2$	628-97-7	$C_{18}H_{36}O_2$	213.1841 115.0730 101.0594	Lipids	Fatty acid ester
3-Nonen-1-ol	7.14	$C_9H_{18}O$	10340-23-5	$C_9H_{18}O$	95.0848 81.0694 67.0540	Lipids	Fatty alcohols

Continuation Supplementary Table 4

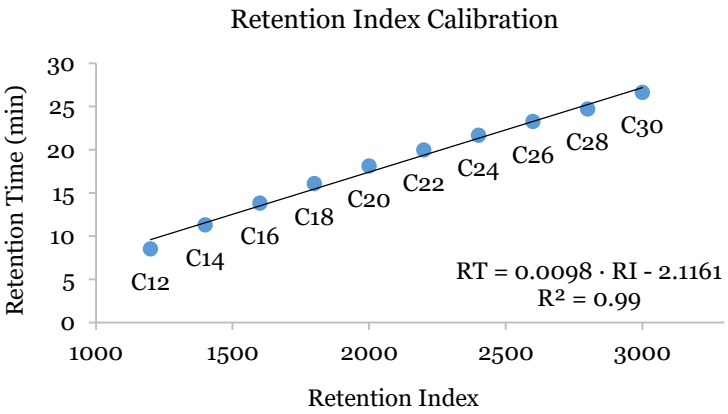
Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
1-Dodecanol	13.40	$C_{12}H_{26}O$	112-53-8	$C_{15}H_{34}OSi$	243.2136 129.0699 97.1002	Lipids	Fatty alcohols
13-Docosenamide	24.59	$C_{22}H_{43}NO$	112-84-5	$C_{22}H_{43}NO$	126.0913 72.0443 59.0364	Lipids	Fatty amide
1-Monoisobutyryl-glycerol	12.13	$C_7H_{14}O_4$		$C_{13}H_{30}O_4Si_2$	203.1091	Lipids	Glycerolipids
1-Monolaurylglycerol	19.92	$C_{15}H_{30}O_4$	27215-38-9	$C_{21}H_{46}O_4Si_2$	315.2347 257.1938 183.1740	Lipids	Glycerolipids
1-Monomyri-stylglycerol	21.56	$C_{17}H_{34}O_4$	589-68-4	$C_{23}H_{50}O_4Si_2$	343.2653 211.2053 129.0590	Lipids	Glycerolipids
2-Monopalmito-ylglycerol	22.83	$C_{19}H_{38}O_4$	23470-00-0	$C_{25}H_{54}O_4Si_2$	313.2552 191.0010 147.0655	Lipids	Glycerolipids
2-Monostearylglycerol	24.27	$C_{21}H_{42}O_4$	621-61-4	$C_{27}H_{58}O_4Si_2$	341.2863 218.1136 191.0851	Lipids	Glycerolipids
1-Monostearylglycerol	24.51	$C_{21}H_{42}O_4$	123-94-4	$C_{27}H_{58}O_4Si_2$	399.3293 341.2868 267.2691	Lipids	Glycerolipids
2-Hydroxyisocaproic acid	8.92	$C_6H_{12}O_3$	498-36-2	$C_{12}H_{28}O_3Si_2$	177.0744 159.1194 103.0548	Lipids	Hydroxy fatty aci
Tridecylic acid	15.49	$C_{13}H_{26}O_2$	638-53-9	$C_{16}H_{34}O_2Si$	271.2093 117.0376 75.0266	Lipids	Long-chain fatty acids
Myristoleic acid	16.31	$C_{14}H_{26}O_2$	544-64-9	$C_{17}H_{34}O_2Si$	283.2080 145.0690 129.0370	Lipids	Long-chain fatty acids
Myristic acid	16.54	$C_{14}H_{28}O_2$	544-63-8	$C_{17}H_{36}O_2Si$	285.2251 145.0681 117.0370	Lipids	Long-chain fatty acids
Pentadecanoic acid	17.55	$C_{15}H30O2$	1002-84-2	$C_{18}H_{38}O_2Si$	314.2593 299.2386 271.2087	Lipids	Long-chain fatty acids
Palmitoleic acid	18.27	$C_{16}H_{30}O_2$	373-49-9	$C_{19}H_{38}O_2Si$	311.2403 185.0990 129.0370	Lipids	Long-chain fatty acids
10-Heptadecenoic acid	19.19	$C_{17}H_{32}O$	29743-97-3	$C_{20}H_{40}O_2Si$	325.2551 250.2277 185.0997	Lipids	Long-chain fatty acids
Arachidonic acid	19.89	$C_{20}H_{32}O_2$	506-32-1	$C_{23}H_{40}O_2Si$	129.0432 117.0382 55.0541	Lipids	Long-chain fatty acids

Continuation Supplementary Table 4

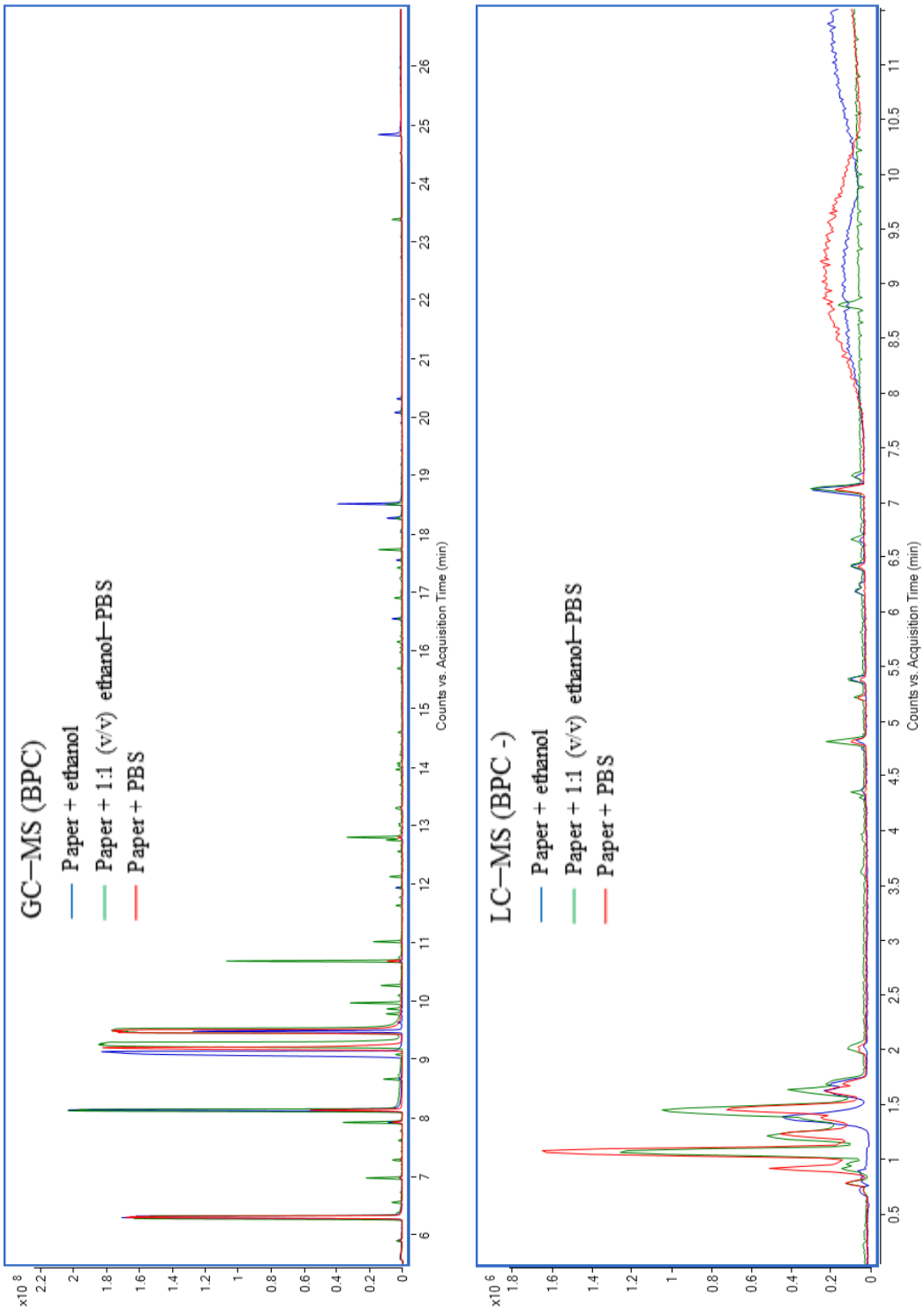
Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
Linoleic acid	19.90	$C_{18}H_{32}O_2$	60-33-3	$C_{21}H_{40}O_2Si$	262.2289 117.0403 67.0535	Lipids	Long-chain fatty acids
Oleic acid	20.08	$C_{18}H_{34}O_2$	112-80-1	$C_{21}H_{42}O_2Si$	339.2723 145.0679 117.0369	Lipids	Long-chain fatty acids
Arachidic acid	21.97	$C_{20}H_{40}O_2$	506-30-9	$C_{23}H_{48}O_2Si$	369.3185 145.0698 129.0380	Lipids	Long-chain fatty acids
Lauric acid	14.39	$C_{12}H_{24}O_2$	143-07-7	$C_{15}H_{32}O_2Si$	257.1934 129.0372 117.0369	Lipids	Medium-chain fatty acid
Cholesta-3,5-diene	25.55	$C_{27}H_{44}$	747-90-0	$C_{27}H_{44}$	368.3437 247.2412 147.1141	Lipids	Steroids
Squalene	24.84	$C_{30}H_{50}$	111-02-4	$C_{30}H_{50}$	367.3350 81.06910 69.06910	Lipids	Terpenoid
Hexacosanoic acid	26.51	$C_{26}H_{52}O_2$	506-46-7	$C_{29}H_{60}O_2Si$	453.4150 409.3558 117.0381	Lipids	Very long-chain fatty acid
Uric acid	18.94	$C_5H_4N_4O_3$	69–93-2	$C_{17}H_{36}N_4O_3Si_4$	456.1680 441.1635 73.0467	Nucleosides, nucleotides, and analogues	Purine
Thymine	11.24	$C_5H_6N_2O_2$	65-71-4	$C_{11}H_{22}N_2O_2Si_2$	270.1205 255.0972 113.0407	Nucleosides, nucleotides, and analogues	Pyrimidine
2-Hydroxyethanamine	5.84	C_2H_7NO		$C_8H_{23}NOSi_2$	190.1066 116.0557 102.0731	Organic nitrogen compounds	Alkanolamines
Ethanolamine	9.40	C_2H_7NO	141-43-5	$C_{11}H_{31}NOSi_3$	174.1133 131.0884 117.0735	Organic nitrogen compounds	Alkanolamines
Diethanolamine	11.71	$C_4H_{11}NO_2$	111-42-2	$C_{10}H_{27}NO_2Si_2$	146.0987 130.0682 59.0310	Organic nitrogen compounds	Alkanolamines
1,3,5-Trihydroxy-tert-butylamine	12.62	C4H10NO3		$C_{16}H_{43}NO_3Si_4$	306.1740 128.0525 102.0731	Organic nitrogen compounds	Alkanolamines
Triethanolamine	14.04	$C_6H_{15}NO_3$	102-71-6	$C_{15}H_{39}NO_3Si_3$	350.2005 262.1647 218.1022	Organic nitrogen compounds	Alkanolamines
Urea	9.18	CON_2H_4	57-13-6	$C_7H_{20}N_2OSi_2$	204.1110 189.0885 171.0781	Organic nitrogen compounds	Urea
2,3-Butanediol	5.85	$C_4H_{10}O_2$	513-85-9	$C_{10}H_{26}O_2Si_2$	147.0653 133.0181 117.0720	Organic oxygen compounds	Alcohols and polyols

Continuation Supplementary Table 4

Compound name	RT (min)	Formula	CAS ID	Formula (derivatization)	Fragments (derivatization product)	Family	Subfamily
4-Methyl-1,2-dihydroxypentane	8.83	$C_6H_{14}O$	24347-54-4	$C_{12}H_{30}O_2Si_2$	159.1192 103.0543 117.0463	Organic oxygen compounds	Alcohols and polyols
Glycerol	9.48	$C_3H_8O_3$	56-81-5	$C_{12}H_{32}O_3Si_3$	218.1146 205.1077 103.0569	Organic oxygen compounds	Alcohols and polyols
1,1,1-Tris(hydroxy-methyl)propane	11.12	$C_6H_{14}O_3$	77–99-6	$C_{15}H_{38}O_3Si_3$	191.0913 157.1036 129.0712	Organic oxygen compounds	Alcohols and polyols
Pinitol	16.25	$C_7H_{14}O_6$	10284-63-6	$C_{22}H_{54}O_6Si_5$	318.1503 260.1260 217.1073	Organic oxygen compounds	Alcohols and polyols
myo-Inositol	18.84	$C_6H_{12}O_6$	87–89-8	$C_{24}H_{60}O_6Si_6$	612.2926 305.1405 217.1060	Organic oxygen compounds	Alcohols and polyols
2-(2-Butoxy-ethoxy)ethanol	10.43	$C_8H_{18}O_3$	112-34-5	$C_{11}H_{26}O_3Si$	117.0713 101.0416 85.0643	Organic oxygen compounds	Ether
Alloxanoic acid	12.48	$C_4H_4N_2O_5$		$C_{16}H_{36}N_2O_5Si_4$	331.1343 172.0963 100.0221	Organoheterocyclic compounds	Diazine
3-Amino-2-piperidone	11.98	$C_5H_{10}N_2O$	42538-31-8	$C_{11}H_{26}N_2OSi_2$	258.1551 243.1327 203.1135	Organoheterocyclic compounds	Lactam
Cyanuric acid	13.79	$C_3H_3N_3O_3$	108-80-5	$C_{12}H_{27}N_3O_3Si_3$	345.1348 215.0685 100.0210	Organoheterocyclic compounds	Triazine
Borate	9.68	H_3BO_3	10043-35-3	$C_9H_{27}BO_3Si_3$	262.1146 224.0792 217.0791	Oxoanionic compound	Miscellaneous metallic oxoanionic compounds

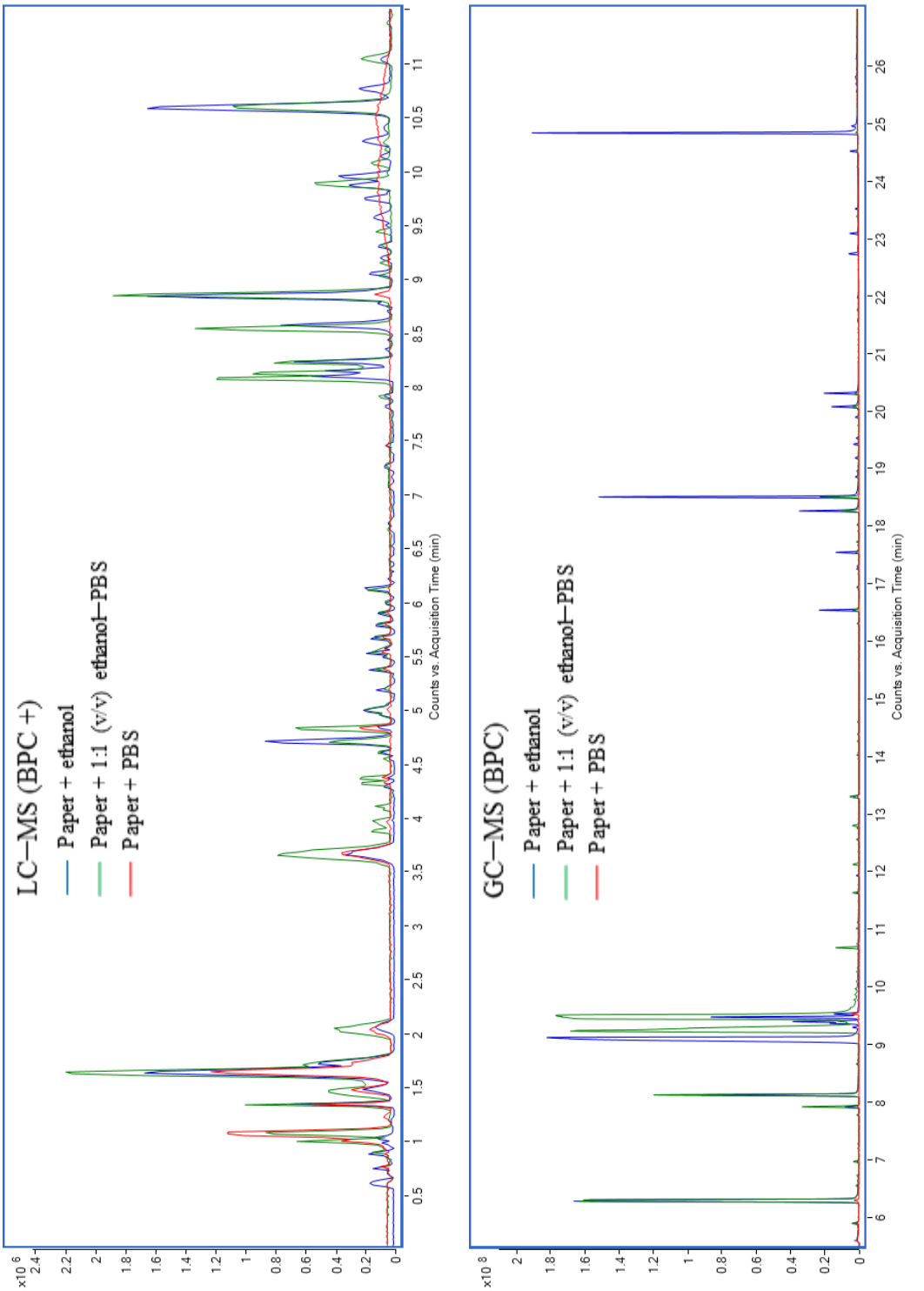


Supplementary Fig. 1. RI calibration line of the alkane standard mixture.

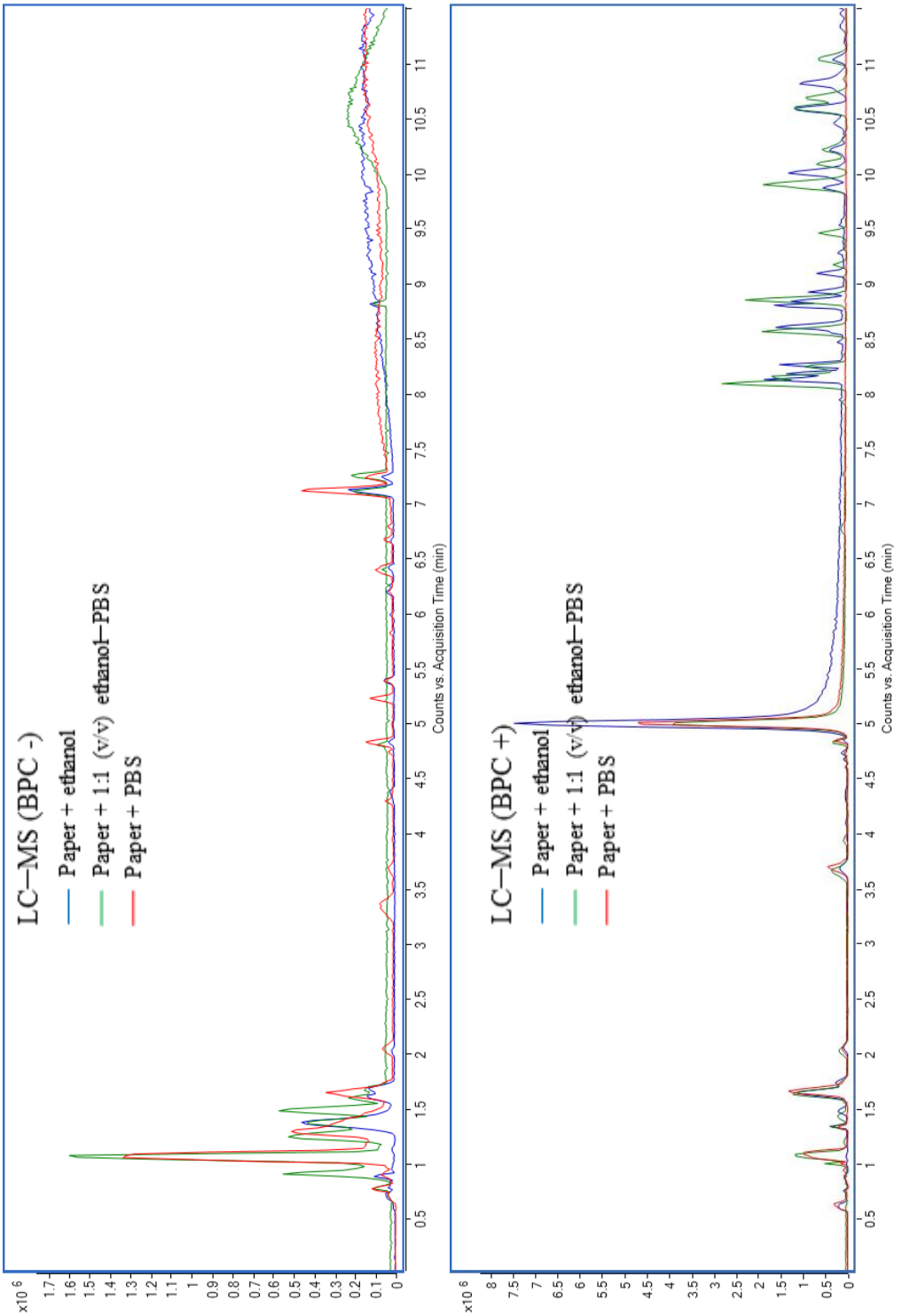


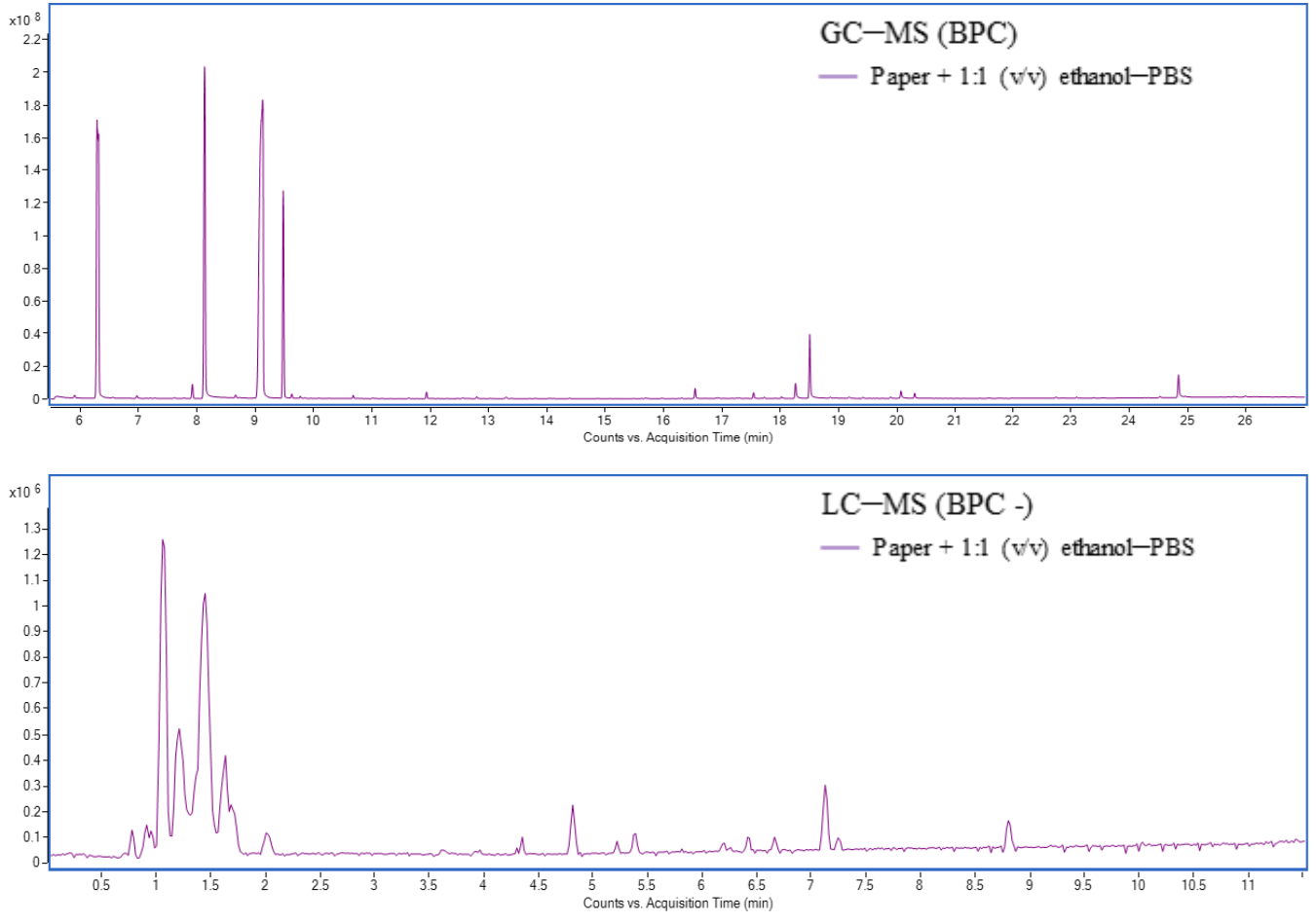
Supplementary Fig. 2. Base peak chromatograms by GC–TOF/MS and LC–QTOF MS/MS obtained from dry sweat collected on filter paper and gauze impregnated with ethanol (blue), 1:1 (v/v) ethanol–PB (green) and PB (red) solutions.

Continuation Supplementary Fig. 2



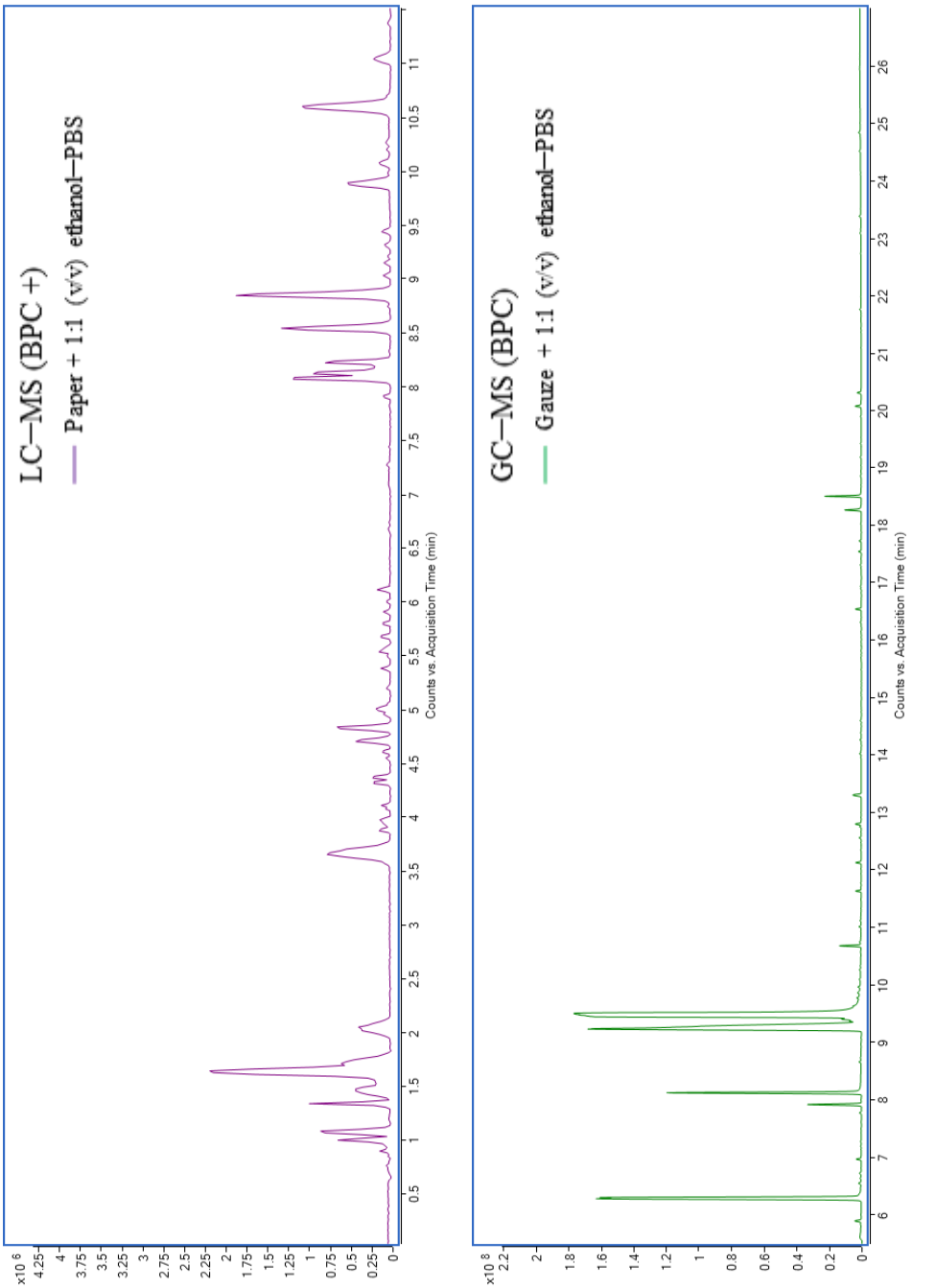
Continuation Supplementary Fig. 2



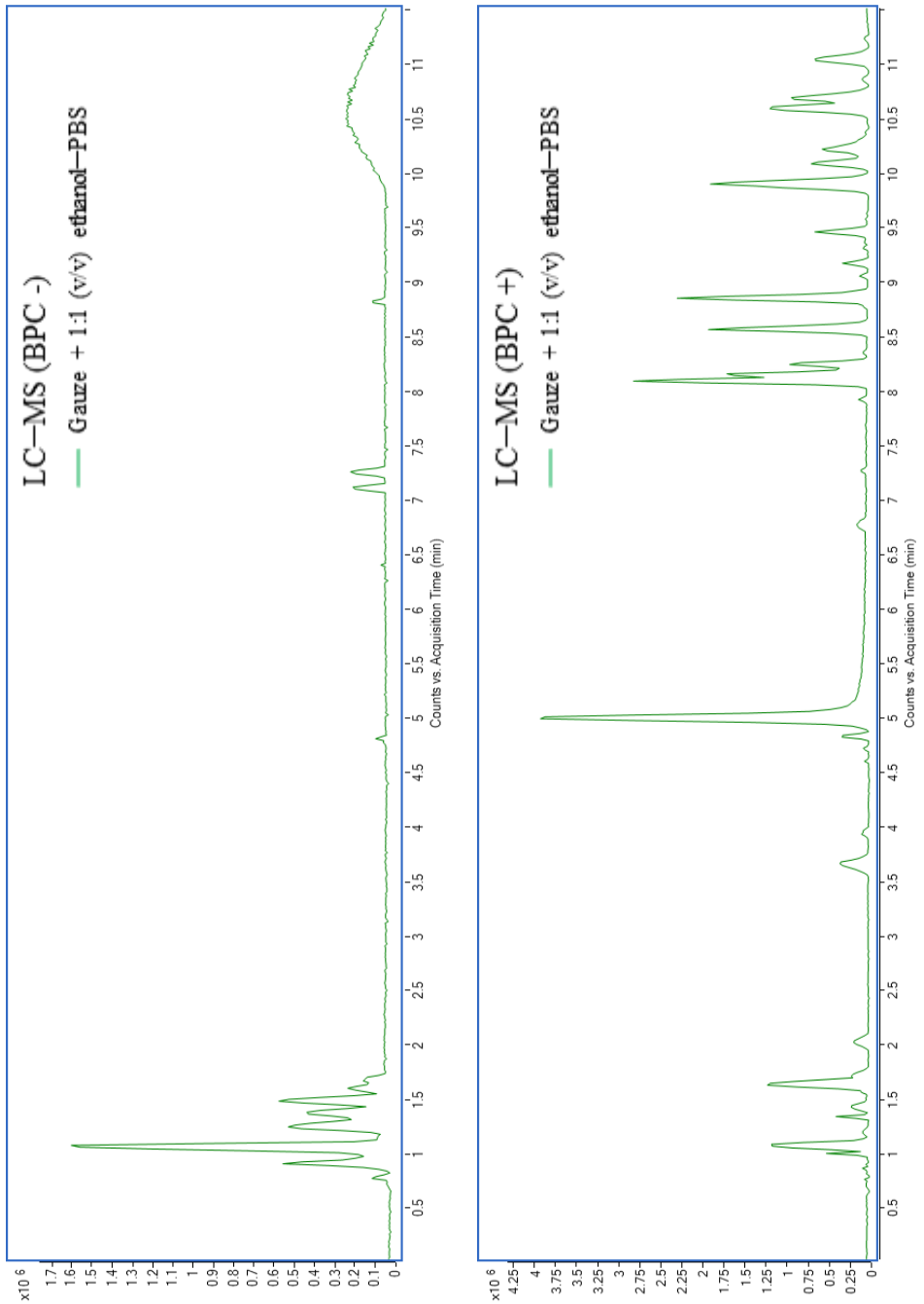


Supplementary Fig. 3. Base peak chromatograms by GC–TOF/MS and LC–QTOF MS/MS obtained from 1:1 (v/v) ethanol–PBS of dry sweat collected by paper (purple) and gauze (green). End region of the base peak chromatogram by LC–QTOF MS/MS (+) obtained from 1:1 (v/v) ethanol–PBS of dry sweat collected on paper, with some of the lipids identified in this region.

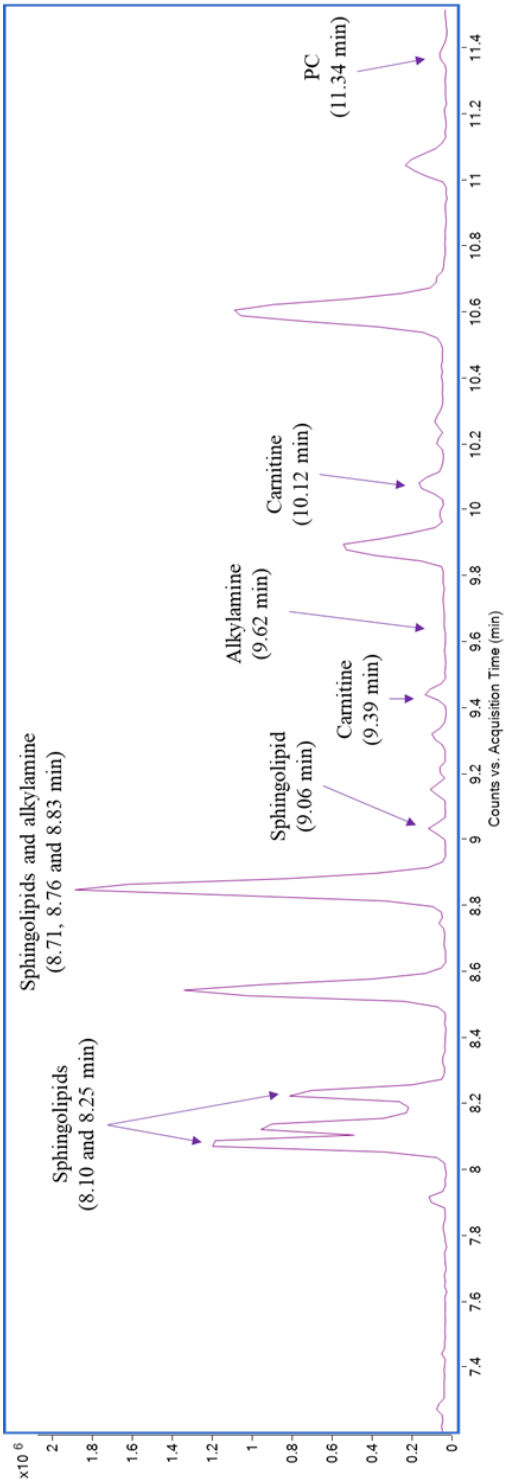
Continuation Supplementary Fig. 3

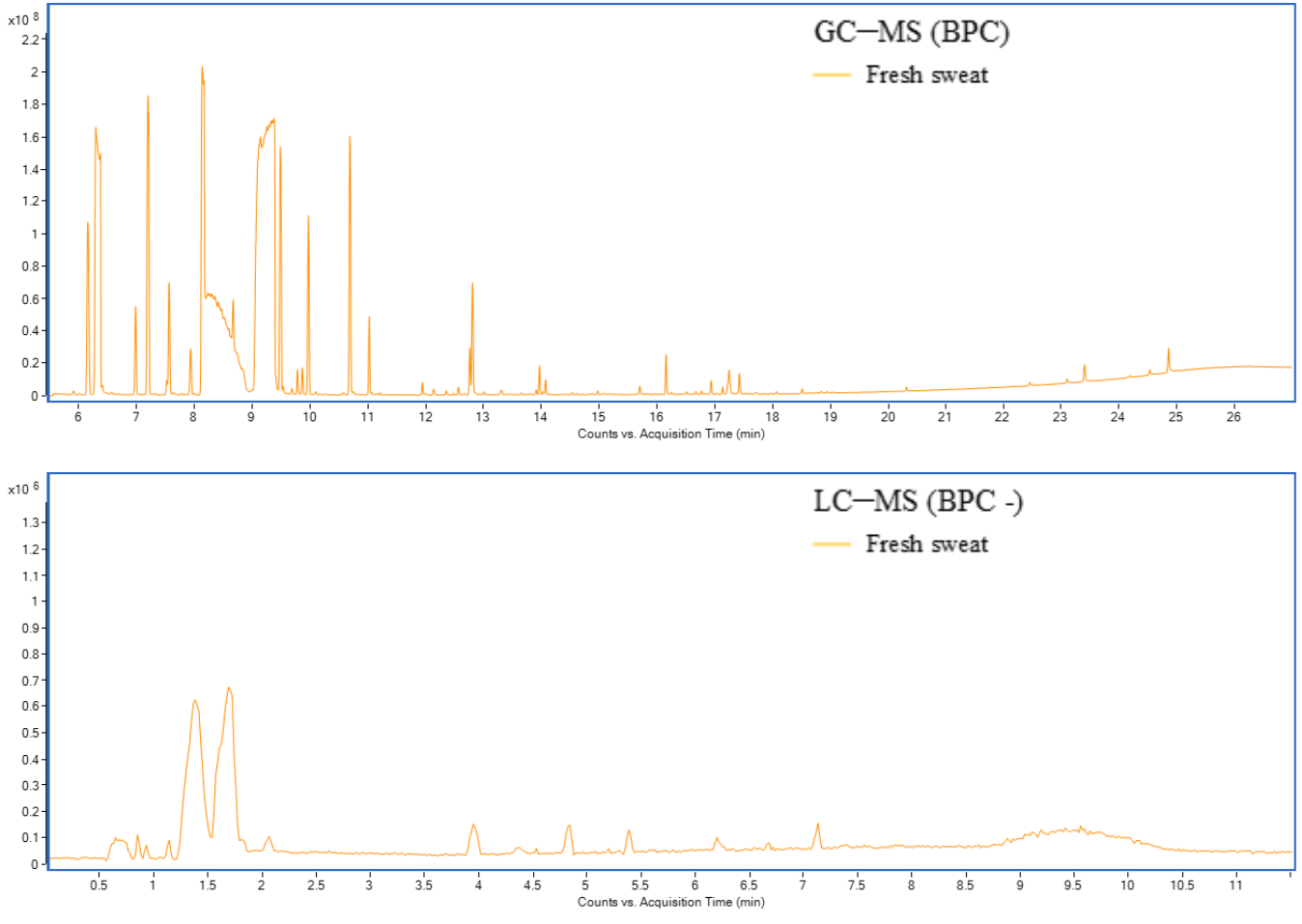


Continuation Supplementary Fig. 3



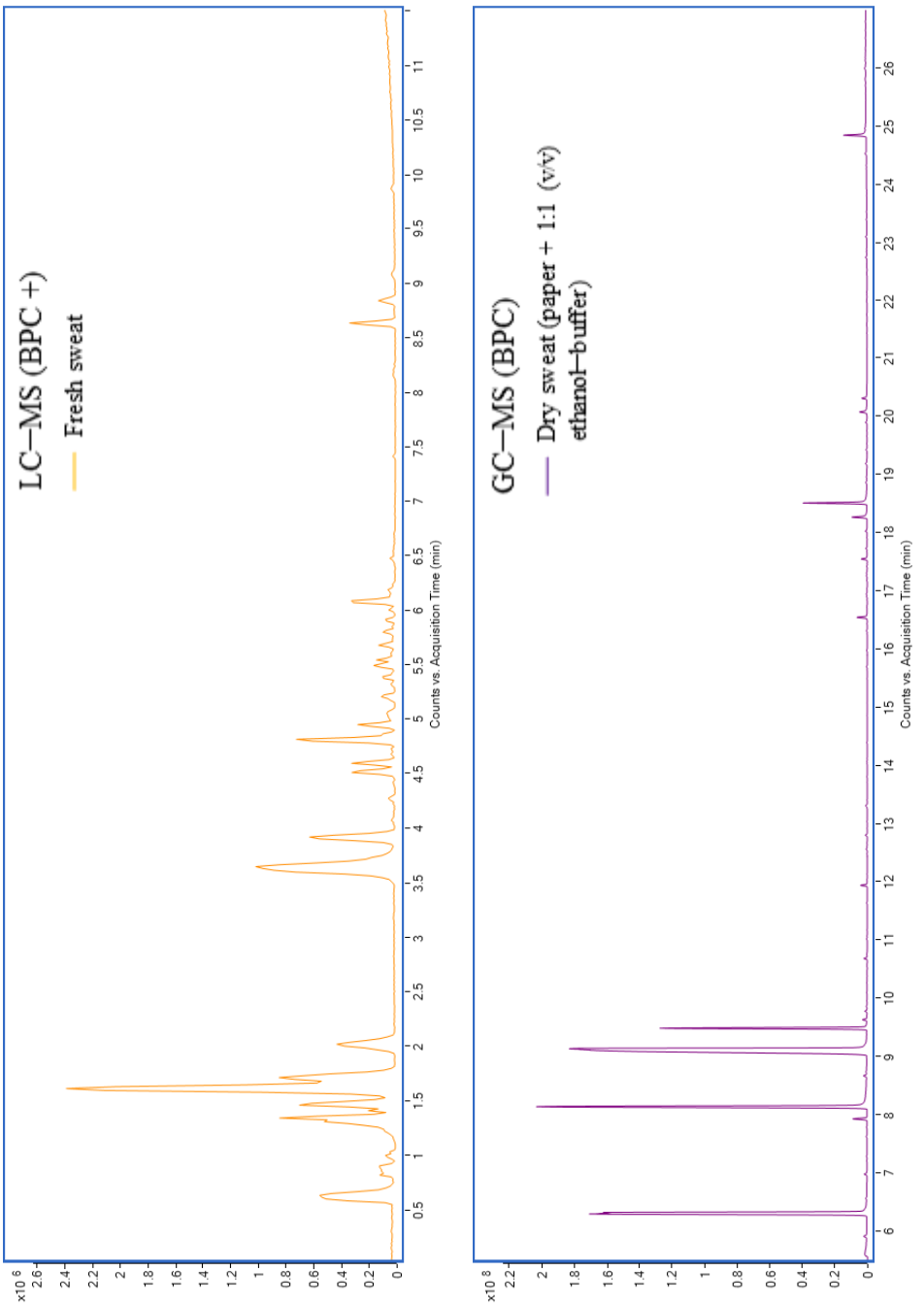
Continuation Supplementary Fig. 3



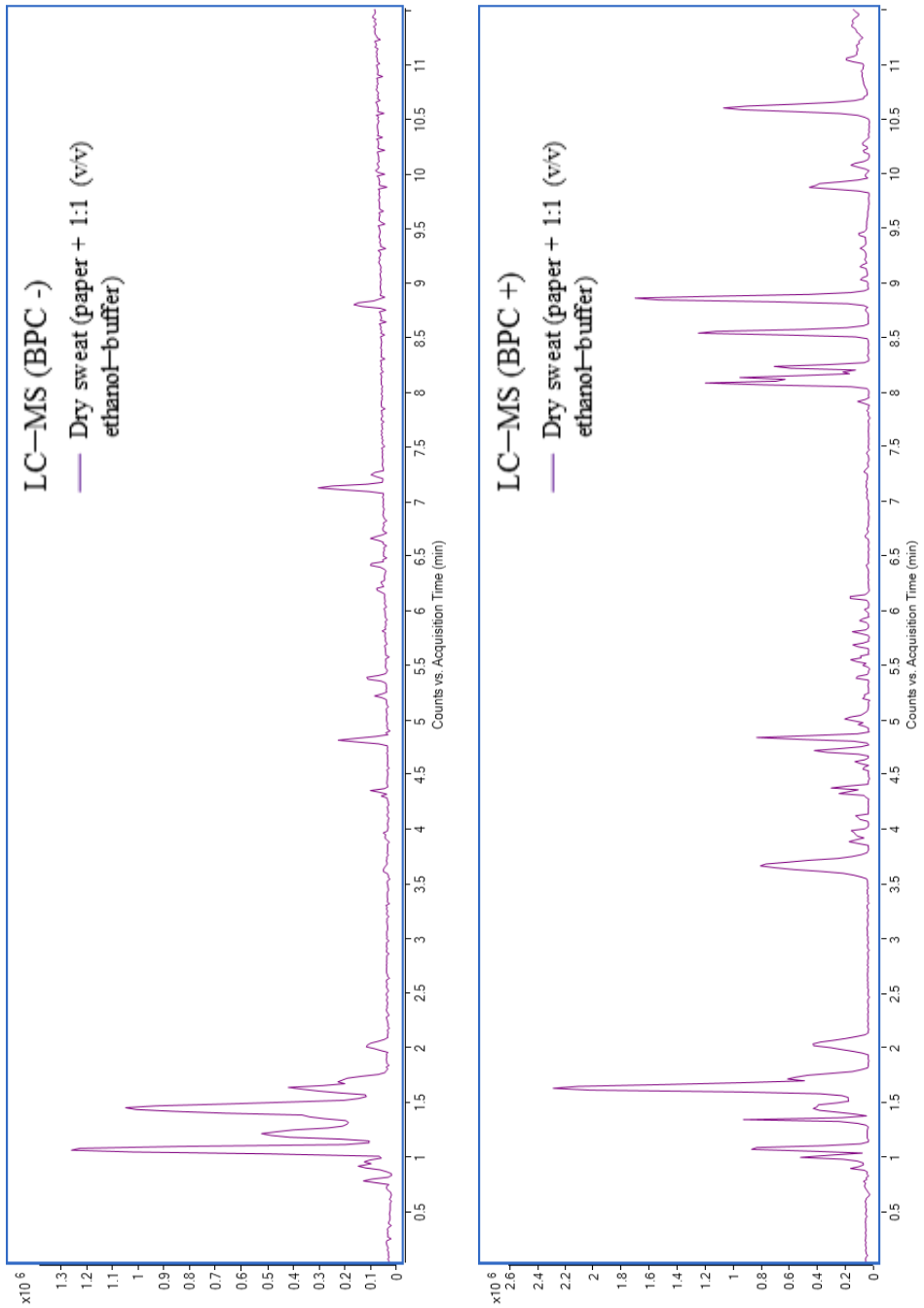


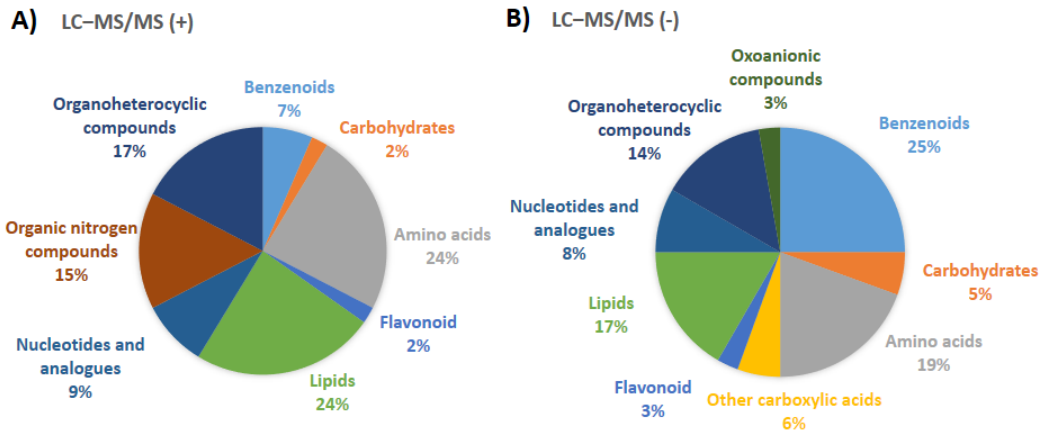
Supplementary Fig. 4. Base peak chromatograms obtained from dry and fresh sweat by GC–TOF/MS and LC–QTOF MS/MS in both ionization modes.

Continuation Supplementary Fig. 4



Continuation Supplementary Fig. 4





Supplementary Fig. 5. Circle graphs comparing the percentage of compounds tentatively identified by LC–MS/MS of each chemical family in dry sweat collected on filter paper impregnated with 1:1 (v/v) ethanol–PBS in positive **(A)** and negative **(B)** ionization modes.

LC–QTOF MS/MS data processing and statistical analysis

MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies) was used to process all data obtained by LC–QTOF in MS/MS mode. Treatment of raw data files started by extraction of potential MFs with the suited algorithm included in the software. For this purpose, the extraction algorithm considered all ions with single charge state exceeding 500 and 600 counts for negative and positive polarities, respectively. These cut-off values were established taking into account the chromatographic background noise. Additionally, the isotopic distribution to consider MFs as valid should be defined by two or more ions (with a peak spacing tolerance of *m/z* 0.0025, plus 7.0 ppm in mass accuracy). Ions and adducts formation in the positive (+H, +Na, +K) and negative ionization (–H, +HCOO, +Cl) modes, as well as the neutral loss by dehydration were included to identify features corresponding to the same potential metabolite. Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities and other contaminants after analysis of a blank for each sample (gauze and filter paper with water, and deionized water) under identical operational conditions for each sample treatment.

Identification of the compounds was supported on MS and MS/MS information and search in the METLIN MS and MS/MS databases (http://metlin.scripps.edu) and the

Human Metabolome Database (HMDB, 4.0 version), using in all cases the MFs obtained in the previous step.

In the next step, the "Batch Targeted Feature Extraction" algorithm from the MassHunter Workstation software (version B6.00 Profinder, Agilent Technologies) was used to extract and align peaks in all injections that matched a database of all identified metabolites. The isotopic distribution for a valid feature had to be defined by two or more ions —with a peak spacing tolerance of 0.0025 *m/z*, plus 7.0 ppm. The features were aligned by using a tolerance window of 0.30 min and a mass accuracy of 10 ppm for retention times (RTs) and *m/z* values across all data files, respectively. Each extracted ion chromatogram was individually examined, and potentially incorrect assignments and integrations were manually verified. The resulting table with the peak area of all identified compounds in the different samples was exported in comma separated value format (.csv file).

GC–TOF/MS data processing and statistical analysis

Unknown Analysis software (version B.07.00, Agilent Technologies) was used to process all data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies) was used to treat all data files. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of MFs considered as potential compounds defined by the *m/z* value of one representative ion for each chromatographic peak and its RT. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions, the accuracy error at 50 ppm and the window size factor at 200 units. Background spectra were subtracted to spectra obtained from each sample before its deconvolution.

Tentative identification of compounds was firstly performed by searching MS spectra in the NIST (version 2.0) database. Only identifications with a match factor and a reverse match factor higher than 700 were considered. The RI values included in the NIST database also supported identifications. For this purpose, an RI calibration model was built by plotting the RTs obtained by analysis of the alkane standard mixture (C10 to C40 with an even number of carbons) with the chromatographic method used in this research and the RI values provided for each alkane by the NIST database. Figure S-1 shows the RI calibration graph and the equation fitting the model. This equation was used to

experimentally estimate the RI value for each identified compound, which must be less than 100 units different as compared with the theoretical value provided by the NIST. This database does not contain high resolution MS information as provided by the TOF detector. For this reason, a third step was included to validate identification of each compound by using high resolution MS. Thus, the molecular formula for the tentative ion $[M]^+$ and the most intense fragments for each MF should fit the NIST identification by setting a cut-off value in mass accuracy of 10 ppm. Identification was also carried out by searching MS spectra on the Fiehn database [19]. Only identifications with a score higher than 70 were considered as valid. The RTs included in the Fiehn database were also considered to support identifications with the requirement for acceptance of a difference between the experimental RT and the theoretical value provided by this database for each target compound below 0.50 min. Additionally, derivatized standards (D-galactose, D-glucose and myo-inositol) were injected for confirmation of some sugars.

Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities and other contaminants after analysis of a blank for each sample (gauze and filter paper with water, and deionized water) under identical operational conditions for each sample treatment procedure used. Finally, the MassHunter Workstation software (version 7.0, Quantitative Analysis, Agilent Technologies) was used to reintegrate all metabolites found in all analyzed samples, using the corresponding characteristic quantifier and qualifiers. The resulting table was exported in comma separated value format (.csv file).

Sudor y metabolómica: Aplicaciones clínicas

Sweat and metabolomics: Clinical applications

In addition to establishing procedures for sampling and sample preparation for the different types of sweat samples, a research was addressed to enhancing the metabolomics coverage of sweat as much as possible. As Chapter X shows, this aim was achieved by a gas chromatography–time of flight/mass spectrometry (GC–TOF/MS) platform in high resolution mode, which provided information for tentative identification of 66 compounds, including amino acids, dicarboxylic acids and other interesting metabolites such as myo-inositol or urocanic acid.

A key contribution of the sweat-metabolomics binomial was the proposal of panels of biomarkers for lung cancer detection, which constitutes Chapter XI. The use of the PanelomiX tool allowed obtaining two panels of sweat metabolites in which both false negatives and false positives were reduced in such a way that 95% was the level reached for both specificity and sensitivity.

Chapter X

Development of a method for enhancing metabolomics coverage of human sweat by gas chromatography–mass spectrometry in high resolution mode

Development of a method for enhancing metabolomics coverage of human sweat by gas chromatography–mass spectrometry in high resolution mode

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Development of a method for enhancing metabolomics coverage of human sweat by gas chromatography–mass spectrometry in high resolution mode

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Abstract

Sweat has recently gained popularity as clinical sample in metabolomics analysis as it is a no invasively sampled biofluid the composition of which could be modified by certain pathologies, as is the case with cystic fibrosis that increases chloride levels in sweat. However, the whole composition of sweat is still unknown and there is a lack of analytical strategies for sweat analysis. The aim of the present study was to develop and validate a method for metabolomic analysis of human sweat by gas chromatography–time of flight/mass spectrometry (GC–TOF/MS) in high resolution mode. Thus, different sample preparation strategies were compared to check their effect on the profile of sweat metabolites. Sixty-six compounds were tentatively identified by the obtained MS information. Amino acids, dicarboxylic acids and other interesting metabolites such as myo-inositol and urocanic acid were identified. Among the tested protocols, methoxymation plus silylation after deproteination was the most suited option to obtain a representative snapshot of sweat metabolome. The intra-day repeatability of the method ranged from 0.60 to 16.99% and the inter-day repeatability from 2.75 to 31.25%. As most of the identified metabolites are involved in key biochemical pathways, this study opens new possibilities to the use of sweat as a source of metabolite biomarkers of specific disorders.

Keywords: Metabolomics, global profiling, human sweat, gas chromatography–mass spectrometry, human sweat metabolome, sample preparation.

1. Introduction

The most common biofluids in metabolomics analysis applied to clinical and nutritional studies are blood (serum or plasma) and urine [1]. Nowadays, alternative biofluids such as sweat [2], tears [3], saliva [4] or exhaled breath [5], with a less complex composition, are gaining popularity as they can be easily obtained in a no invasive manner [2,6]. Among them, sweat is a biofluid produced by the eccrine and apocrine sweat glands located in the epidermis. The primary function of sweating is thermoregulation to control body temperature by evaporative cooling. In addition, sweat is a defense mechanism of skin, the excretion fluid of chemosignals such as androstadienone —which acts as hormonal stimuli in females—, and waste of metabolites such as uric acid [7]. Sweat is mainly composed by water (99%) containing electrolytes, small molecules as amines, carboxylic acids and amino acids, more complex biomolecules such as proteins and antimicrobial peptides, and xenobiotics such as drugs, cosmetics, and ethanol, among others [8]. Except for the case of proteins [9], most sweat components are small molecules resulting from metabolic pathways; therefore, their study pertains to the metabolomics field. Diseases can change sweat composition either by altering the concentration of common components or reporting new components that, in any case, could act as tentative biomarkers of the given disease. Therefore, the varied composition of sweat supports its clinical interest to be potentially exploited for diagnostic.

The scant traditional use of sweat as clinical sample is explained by both the lack of studies to relate sweat composition with pathological states, and the absence of reproducible sweat collectors. In fact, few clinical tests use sweat as sample. One of them, already well implemented, is cystic fibrosis diagnosis in new-borns, based on the determination of chloride in sweat [10,11]. Recently, a new indicator test (Neuropad) has been developed to detect diabetes in foot sweat [12], since recent studies have found a credible biomarker that shows a strong correlation between sweat glucose and blood glucose, providing sweat is properly obtained to prevent contamination of the skin surface from other sources of glucose [13]. Thus, the advances both in sweat collection devices and sensitive analytical techniques increased the interest on sweat testing of drugs over the past few years [14–18]. Indeed, sweat is, together with urine, the preferred sample for doping control, each biofluid with its advantages and limitations. Sweat allows a no invasive sampling for continuous monitoring of drugs exposure as the samples can be collected for a programmed period with minimal disturbance for the sampled individual. Sweat

sampling can be performed with the aid of sweat wipes as liquid perspiration or over time using sweat patches [19,20]. The experience gained in sweat collection procedures for the analysis of xenobiotics could help to implement this biofluid in clinical diagnostics [12,21]. No invasive sampling, avoidance of infections risk to patients who need daily analysis, and absence of requirements in terms of health personnel care are the main benefits from sweat as clinical sample.

The main limitations of sweat as clinical sample are the difficulty to produce enough sweat for analysis, the variability in secretion among individuals [22,23], and the problems associated to sample representativeness and presence of interferents. Despite the scant studies on the clinical perspectives of sweat, the potential of this biofluid in omics disciplines has been pointed out. In proteomics, a current research has revealed that sweat proteome is rather different from serum proteome [9]. Therefore, human sweat could be considered an additional source of unique disease-related biomolecules —in fact, differential abundances of selected proteins have been found in sweat from schizophrenia patients and control individuals [9]. This preliminary test should be validated to prove the applicability of human sweat in the diagnostic of schizophrenia. In the metabolomics field, a recent study on the composition of human sweat by high-resolution NMR spectroscopy has revealed that the main constituents found in human sweat from healthy people are compounds involved in primary and secondary biological functions [24]. Among them, amino acids, sugars, lactate, glycerol, and compounds involved in the citric acid cycle (*e.g.*, pyruvate, fumarate or aconitate) have been detected. NMR is especially suited to metabolomics profiling of human sweat as this is a relatively no complex biofluid. Other recent study of sweat was carried out by liquid chromatography–quadrupole time of flight–tandem mass spectrometry (LC–QTOF MS/MS) in high resolution mode, which has confirmed that most of human sweat components are involved in key biochemical pathways [2]. Forty-one compounds were identified by MS/MS. Among them, amino acids, dicarboxylic acids and other interesting metabolites were detected. Other study of untargeted analysis of sweat by LC–QTOF MS/MS revealed metabolomic discrimination between subjects with and without lung cancer [25]. A recent study of sweat was carried out by Fourier-transform ion cyclotron resonance mass spectrometry, in which only eight compounds were identified by MS/MS [20].

More research on sweat composition is demanded to assess the potential of this biofluid for clinical diagnostic as, at present, only NMR and LC–MS have been used for

sweat characterization. So, the aim of the present study was to develop and validate a method for analysis of human sweat by GC–TOF/MS. The use of this approach is desirable because it allows studying a wide variety of compounds. Furthermore, human sweat is composed by volatile and no volatile compounds, and GC–TOF/MS allows detecting both types of compounds after applying wellknown derivatization reactions for the latter. Different sample preparation strategies were compared to check their influence on the profile of the detected metabolites. Identification of metabolites by GC–TOF/MS was carried out to obtain a snapshot of the composition of sweat metabolome.

2. Experimental

2.1. Reagents

*Trace*SELECT® grade acetonitrile, dichloromethane, ethyl acetate, methanol and *n*-hexane from Sigma–Aldrich (St. Louis, MO, USA) were used for standards and sample preparation. Deionized water (18 mΩ cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used for standards preparation. Pyridine from Merck (Darmstadt, Germany) and methoxyamine hydrochloride from Sigma–Aldrich were used as solvent and reagent, respectively, for methoxymation. Bis-(trimethylsilyl) fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were used as silylation agents in the second derivatization step. MS-grade perfluorotri-*n*-butylamine (PFTBA) from Agilent Technologies (Santa Clara, CA, USA) was used for daily mass calibration. A standard mixture containing ten linear alkanes from C10 to C40 designed for performance tests in GC from Sigma–Aldrich was used to establish the retention index (RI) calibration model. Standards of glutamic acid, phenylalanine, proline, D-galactose, D-glucose, myo-inositol, phosphate, *p*-hydroxybenzoic, fumaric, malic, succinic, caprylic, lauric and palmitic acids from Sigma–Aldrich were used to confirm identification of amino acids, benzene derivatives, dicarboxylic acids, fatty acids and sugars. UV–grade *n*-hexane and acetone from Scharlab (Barcelona, Spain) were used for washing the injection syringe.

2.2. Instruments and apparatus

An IKA® vortex shaker from IKA (Wilmington, NC, USA) was used to promote deproteination or liquid–liquid extraction (LLE). A Concentrator plus™ from Eppendorf (Hamburg, Germany) with three operation modes (vacuum concentrator, centrifuge or dessicator) and three application modes (aqueous, alcohol or high vapor pressure) was used to centrifuge and evaporate the analytical sample before derivatization. A block heater from Stuart Equipment (Staffordshire, ST15 OSA, UK) was used in the derivatization step.

An Agilent 7890A Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with electron impact (EI) source (Santa Clara, CA, USA) was used for analysis. The analytical sample was thus monitored in high resolution mode. MassHunter GC–QTOF Acquisition software (version B.06, Agilent Technologies) was used to control data acquisition and set the parameters for optimum operation.

2.3. Sample production and collection

The Macroduct® Sweat Analysis System (Wescor, Utah, USA) used for sampling consists of a Webster sweat inducer and a Macroduct sweat collector (US Patent 4,542,751). Pilogel®discs (US Patent 4,383,529) (Wescor, Utah, USA) consisting of a gel reservoir of pilocarpinium ions were used in the iontophoretic stimulation of sweat excretion. The sweat inducer provides a current intensity of 1.50 mA for 5 min through two Pilogel discs as electrodes located on the forearm. Previously, the skin was cleaned with ethanol and then with distilled water. The Macroduct device collected sweat for 15 min, which was transferred to plastic microEppendorf tubes and stored at −80 ºC until analysis. A total sweat volume of at least 70 µL was collected per individual.

Sweat samples for development and characterization of the method were kindly donated by healthy volunteers at Reina Sofía University Hospital (Córdoba, Spain). Pools of the biofluid used for optimization of the method were prepared by mixing aliquots from six healthy volunteers (2 men and 4 women). All subjects gave their informed consent for sweat sampling. No restrictions about diet or age were taken into account. All steps from sweat sampling to analysis were carried out in accordance with the ethical principles of human medical research (World Medical Association, Helsinki Declaration, 2004). The study was approved by the ethical committee of the Reina Sofia University Hospital.

2.4. Sample treatment

A pool was prepared taking 70 μL of samples from each participant. Then, three experimental strategies were tested to select that providing maximum metabolite coverage. (i) Deproteination with methanol–acetonitrile before methoxymation plus silylation. With this aim, a 25 μL aliquot was vortexed with 150 μL of 70:30 methanol–acetonitrile in a glass insert at room temperature for 5 min, then centrifuged for 5 min at 10000×*g*. The liquid phase was isolated and located into a new glass insert, evaporated to dryness and reconstituted by 20 μL of 40 mg/mL methoxyamine hydrochloride in pyridine. After adding the methoxymation agents, the mixture was shaken and maintained at 30 °C for 90 min. Then, for silylation, 90 μL of an 100:1 BSTFA–TMCS mixture was added, shaken for 30 s and maintained at 37 °C for 30 min. (ii) Extraction with dichloromethane before methoxymation and silylation. With this aim, a 25 μL aliquot was vortexed with 150 μL of diclorомethane in a glass insert at room temperature for 5 min and centrifuged for 5 min at 10000×*g*. Then, the organic phase was isolated and located into a new glass insert for evaporation to dryness before methoxymation plus silylation. (iii) Extraction with ethyl acetate before methoxymation plus silylation. In this case, a 25 μL aliquot was vortexed with 150 μL of ethyl acetate in a glass insert at room temperature for 5 min and centrifuged for 5 min. Then, the organic phase was isolated and located into a new glass insert for evaporation to dryness before methoxymation plus silylation. All samples were cooled at room temperature for subsequent analysis.

The samples thus prepared were analyzed directly and also after 1:5 dilution with hexane to cover both concentrated and diluted metabolites.

2.5. GC–TOF/MS analysis

GC–TOF/MS analyses were performed by EI ionization mode at 70 eV and controlled by MassHunter Acquisition B.06. Chromatographic separation was carried out with a fused silica DB-5MS-UI 30 m×0.25 mm i.d.×0.25 μm film thickness capillary column from Agilent Technologies. The GC oven temperature program started at 60 °C (1 min held), followed by a temperature ramp of 10 °C/min to final 300 °C (2 min held). A post-run time was programmed for 4 min up to 310 °C to assure complete elution of the injected sample. One μL of sample was injected using pulsed split injection (1:10 split ratio) at 250 °C (the data in the Fiehn library are obtained with this injection mode, and the

sensitivity of the proposed method is enough for using this injection mode). Ultrapure grade helium was used as carrier gas at 1 mL/min. The transfer line, ion source and quadrupole temperatures were set at 280, 300 and 200 °C, respectively. A solvent delay of 5.50 min was used to prevent damage of the ion source filament. The TOF detector was operated at 5 spectra/s in the mass range *m/z* 40–750 and the resolution was 8500 (full width half maximum, FWHM) at *m/z* 501.9706. Daily mass calibration was performed with PFTBA.

2.6. Identification of metabolites

Identification was firstly carried out by searching MS spectra in the NIST11 database. Only identifications with a match factor and a reverse match factor higher than 700 were considered. Furthermore, the RI values included in the NIST database were used to support identifications. For this purpose, an RI calibration model was built by plotting the retention times (RT) obtained by analysis of the alkane standard mixture (C10 to C40 with an even number of carbons) with the chromatographic method used in this research and the RI values provided for each alkane by the NIST database. This equation was used to estimate the RI value for each identified compound, which has to be less than 100 units different as compared with the theoretical value provided by the NIST. This database does not contain high resolution MS information as provided by the TOF detector. For this reason, a third step was included to validate identification of each compound by using high resolution MS. Thus, the molecular formula for the tentative ion $[M]^+$ and the most intense fragments for each molecular feature (MF) should fit the NIST identification by setting a cut-off value in mass accuracy of 10 ppm.

Identification was also carried out by searching MS spectra on the Fiehn database [26]. Only identifications with a score higher than 70 were considered as valid. The RT included in the Fiehn database were also taken into account to support identifications with the requirement for acceptance of a difference between the experimental RT and the theoretical value provided by this database for each target compound below 0.50 min.

Additionally, derivatized standards (glutamic acid, phenylalanine, proline, D-galactose, D-glucose, myo-inositol, phosphate, *p*-hydroxybenzoic, fumaric, malic, succinic, caprylic, lauric and palmitic acids) were injected for confirmation of some amino acids, sugars, benzene derivatives, dicarboxylic and fatty acids.

2.7. Data processing and statistical analysis

Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to unzip all data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by GC–TOF/MS in full scan mode. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of MFs considered as potential compounds defined by the *m/z* value of one representative ion for each chromatographic peak and its RT. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions exceeding 1500 counts for the absolute height parameter, the accuracy error at 50 ppm and the window size factor at 150 units. The list of MFs obtained for each analysis was exported as data files in compound exchange format (.cef files). Tentative identification of compounds was performed by searching each mass spectrum in the NIST 11 and Fiehn databases using the RI or RT value, respectively.

3. Results and discussion

Sweat samples are not ready for direct analysis by GC–MS; therefore, a sample preparation step is demanded to define suited protocols to cover the maximum number of metabolites to characterize sweat composition in a single analysis.

3.1. Optimization of sample preparation

3.1.1. Sample clean-up

Deproteination and liquid–liquid extraction (LLE) can be good sample preparation strategies for cleaning sweat sample prior to GC–MS analysis thanks to their efficiency. Thus, taking into account the chemical diversity of compounds present in sweat, the performance of two different extractants (ethyl acetate and dichloromethane) and a deproteination protocol using methanol–acetonitrile were compared. Both extractants have different polarity and they are water immiscible, which seemed to be desirable for further comparison of the obtained extracts. In LLE, aliquots of the sweat pool were 1:6

extracted with each solvent and the resulting extracts analyzed by GC–TOF/MS. The same ratio was used for deproteination, in which a 70:30 (v/v) methanol–acetonitrile mixture was added to sweat pool aliquots. In the three cases the same generic derivatization protocol that encompasses methoxymation plus silylation was used prior to injection. Supplementary Fig. 1 shows the base peak chromatograms (BPCs) provided by analysis of each extract as well as for the deproteinized sample. Some signals ascribed to column bleeding were detected in the chromatograms, but they were easily identified and discarded prior to comparison among extracts and the deproteinized sample.

Fig. 1A shows the Venn diagram comparing the compounds tentatively identified in the extracts and the deproteinized sample. The compounds list is shown in Supplementary Table 1. As can be seen, deproteination enabled the detection of 62 compounds, while only 24 and 28 of them were extracted with ethyl acetate and dichloromethane, respectively. As all the compounds detected in the extracts were also found in the deproteinized sample, deproteination was concluded as the most adequate strategy for sweat preparation prior to untargeted GC–MS analysis. The low extraction efficiency of LLE was ascribed to the wide variety of sweat components, which makes impossible to find an extractant with capability to isolate compounds pertaining to different chemical families. In fact, the extracts contained less than 50% of the sweat components detected with deproteination, and only in some cases the extraction was quantitatively efficient as compared to deproteination.

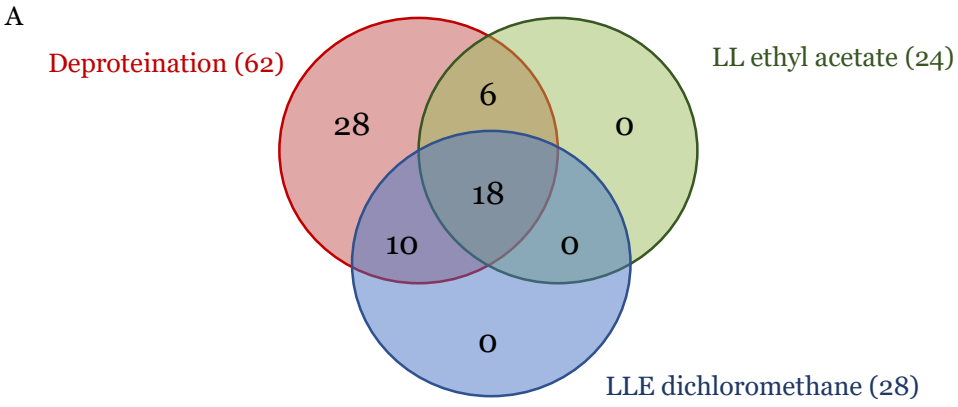

Fig. 1. (A) Venn diagram comparing the number of compounds identified in sweat extracts (ethyl acetate and dichloromethane) as well as in sweat deproteneized with methanol–acetonitrile.

B

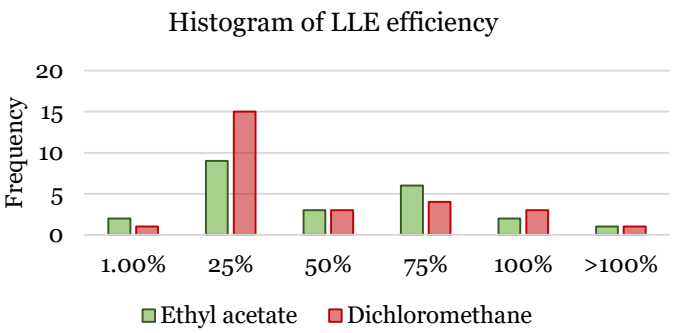

Fig. 1. (B) Histogram representing the number of metabolites detected in ethyl acetate and dichloromethane extracts taking as 100% those obtained by deproteination.

Only nonanoic acid presented a lower area in deproteinized samples as compared to the two extracts, while the rest of metabolites were not efficiently extracted, as can be seen in Fig. 1B. Three metabolites —hexanoic, glycolic and oxalic acids— were extracted by dichloromethane with an efficiency higher than 90% as compared to the protein precipitation protocol, while only hexanoic acid and phenol were efficiently isolated with ethyl acetate as compared to the area obtained by the protein precipitation protocol.

3.1.2. Derivatization

As mentioned above, a standard derivatization procedure consisting of a methoxymation followed by silylation was selected as a first approach. This is the most common derivatization protocol used in metabolomic studies based on GC–MS, in which the methoxymation step is sometimes avoided to simplify the protocol, since the decisive step for increasing volatility and enhance metabolite detection is silylation. On the other hand, methoxymation involves the replacement of the oxygen in a carbonyl group (aldehyde or ketone) by methoxyamine, thus protecting these carbonyl groups from silylation to assure metabolite identification. However, not all aldehyde or ketone groups are protectable; in fact, carbonyl groups adjacent to heteroatoms, such as nitrogen or oxygen, are not electropositive enough to become target of nucleophilic attack by the methoxyamine reagent. Therefore, the methoxymation step does not alter some kind of metabolites, thus being optional or unnecessary in some cases. The removal of this step not only reduces the derivatization time, but also gives more stability to the final products. With

these premises, derivatization protocols with and without methoxymation before silylation were tested. Fig. 2 shows the Venn diagram comparing the compounds identified in both cases, which are listed in Supplementary Table 2**.** As can be seen, the removal of the methoxymation step reduces the number of identified metabolites from 62 to 58, which is the result of the loss of 8 metabolites no identified without methoxymation, and the appearance of 4 new metabolites only detected without methoxymation. Three of the identified sugars (galactose, glucose and maltose) were detected with and without methoxymation, giving different peaks after each derivatization protocol. Going deeper into the chemical structure of the identified metabolites, more than a half (57%) of the compounds with aldehyde or keto groups were wrongly identified when the methoximation step was not carried out. Therefore, methoxymation plus sylilation after deproteination with methanol–acetonitrile seems to be the suited sample preparation approach for sweat analysis by GC–TOF/MS.

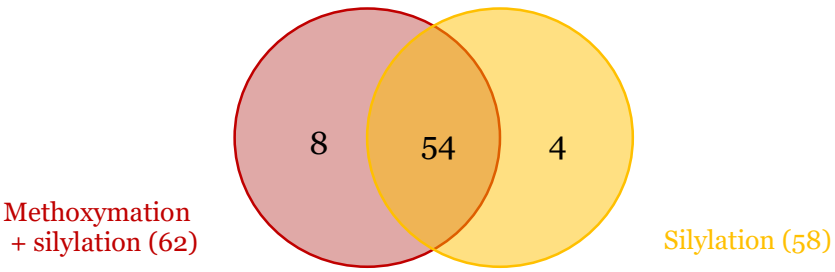

Fig. 2. Venn diagram comparing the number of compounds identified in sweat after deproteination following a derivatization step with and without methoxymation prior to silylation.

3.2. Identification of sweat compounds detected by GC–TOF/MS

A total of 66 compounds were identified in sweat (including fatty acids, amino acids, dicarboxylic acids, sugars and benzene derivatives, among others) using the tested protocols. This wide variety of compounds supports the interest of this biofluid in clinical analysis. Table 1 lists the identified compounds classified according to their chemical family.

Table 1. Compounds identified in sweat by GC–TOF/MS, classified by the chemical family.

Compound	RT (min)	Formula	CAS ID	Formula (derivatization product)	Fragments (derivatization product)	Family
Phenol	6.22	C_6H_6O	108-95-2	$C_9H_{14}OSi$	166.0801 – $[C_9H_{14}OSi]^+$ 151.0565 – $[C_8H_{11}OSi]^+$ 135.0257 – $[C_7H_7OSi]^+$	Benzene derivatives (alcohols)
Benzoic acid	9.17	$C_7H_6O_2$	65-85-0	$C_{10}H_{14}O_2Si$	194.0739 – $[C_{10}H_{14}O_2Si]^+$ 179.0515 – $[C_9H_{11}O_2Si]^+$ 105.0325 – $[C_7H_5O]^+$	Benzene derivatives (acids)
4-Hydroxybenzoic acid	14.10	$C_7H_6O_3$	99-96-7	$C_{13}H_{22}O_3Si_2$	282.1083 – $[C_{13}H_{22}O_3Si_2]^+$ 267.0863 – $[C_{12}H_{19}O_3Si_2]^+$ 223.0965 – $[C_{11}H_{19}OSi_2]^+$	Benzene derivatives (acids)
Isopropyl 4-hydroxybenzoate	14.55	$C_{10}H_{12}O_3$	4191-73-5	$C_{13}H_{20}O_3Si$	252.1168 – $[C_{13}H_{20}O_3Si]^+$ 237.0935 – $[C_{12}H_{17}O_3Si]^+$ 210.0706 – $[C_{10}H_{14}O_3Si]^+$	Benzene derivatives (esters)
Glycerol	9.52	$C_3H_8O_3$	56-81-5	$C_{12}H_{32}O_3Si_3$	293.1401 – $[C_{11}H_{29}O_3Si_3]^+$ 218.1127 – $[C_9H_{22}O_2Si_2]^+$ 205.1071 – $[C_8H_{21}O_2Si_2]^+$	Carbohydrates (sugar alcohols)
Glyceric acid	10.30	$C_3H_6O_4$	473-81-4	$C_{12}H_{30}O_4Si_3$	307.1189 – $[C_{11}H_{27}O_4Si_3]^+$ 292.1329 – $[C_{11}H_{28}O_3Si_3]^+$ 189.0760 – $[C_7H_{17}O_2Si_2]^+$	Carbohydrates (sugar acids)
Threonic acid	13.04	$C_4H_8O_5$	7306-96-9	$C_{16}H_{40}O_5Si_4$	292.1326 – $[C_{15}H_{24}O_2Si_2]^+$ 220.0931 – $[C_8H_{20}O_3Si_2]^+$ 205.1057 – $[C_{12}H_{17}OSi]^+$	Carbohydrates (sugar acids)
Erythrulose	14.63	$C_4H_8O_4$	496-55-9	$C_{16}H_{41}NO_4Si_4$	320.1512 – $[C_{16}H_{26}NO_2Si_2]^+$ 117.0641 – $[C_4H_{11}NOSi]^+$ 103.0472 – $[C_3H_9NOSi]^+$	Carbohydrates (monosaccharides)
1,5-Anhydro-D-sorbitol	16.53	$C_6H_{12}O_5$	154-58-5	$C_{18}H_{44}O_5Si_4$	259.1165 – $[C_{16}H_{33}O_2Si]^+$ 217.1061 – $[C_{10}H_{17}O_5]^+$ 191.0894 – $[C_{11}H_{15}OSi]^+$	Carbohydrates (monosaccharides)
Sorbose	16.72	$C_6H_{12}O_6$	3615-56-3	$C_{22}H_{55}NO_6Si_5$	307.1560 – $[C_{12}H_{31}O_3Si_3]^+$ 277.1447 – $[C15H25OSi_2]^+$ 217.1060 – $[C_9H_{21}O_2Si_2]^+$	Carbohydrates (monosaccharides)
Psicose	16.82	$C_6H_{12}O_6$	551-68-8	$C_{22}H_{55}NO_6Si_5$	307.1560 – $[C_{12}H_{31}O_3Si_3]^+$ 277.1447 – $[C_{15}H_{25}OSi_2]^+$ 217.1060 – $[C_9H_{21}O_2Si_2]^+$	Carbohydrates (monosaccharides)
Galactose	16.91	$C_6H_{12}O_6$	3646-73-9	$C_{21}H_{52}O_6Si_5$	305.1384– $[C_{16}H_{25}O_2Si_2]^+$ 265.1097– $[C_{10}H_{21}O_6Si]^+$ 204.0998– $[C_8H_{20}O_2Si_2]^+$	Carbohydrates (monosaccharides)
	17.19			$C_{22}H_{55}NO_6Si_5$	319.1559 – $[C_{17}H_{27}O_2Si_2]^+$ 205.1053 – $[C_{12}H_{17}OSi]^+$ 160.0764 – $[C_6H_{14}NO_2Si]^+$	
Methyl β-D-galactopyranoside	16.95	$C_7H_{14}O_6$	3396-99-4	$C_{19}H_{46}O_6Si_4$	217.1068 – $[C_9H_{21}O_2Si_2]^+$ 204.0998 – $[C_8H_{20}O_2Si_2]^+$ 191.0914 – $[C_7H_9O_2Si_2]^+$	Carbohydrates (monosaccharides)
Glucose	16.98	$C_6H_{12}O_6$	50-99-7	$C_{22}H_{55}NO_6Si_5$	319.1563 – $[C_{13}H_{31}O_3Si_3]^+$ 205.1065 – $[C_8H_{21}O_2Si_2]^+$ 160.0784 – $[C_6H_{14}NO_2Si]^+$	Carbohydrates (monosaccharides)
	17.78			$C_{21}H_{52}O_6Si_5$	435.1960 – $[C_{21}H_{39}O_2Si_4]^+$ 305.1449 – $[C_{11}H_{33}Si_5]^+$ 204.1752 – $[C_{10}H_{28}Si_2]^+$	
Myo-Inositol	18.89	$C_6H_{12}O_6$	87-89-8	$C_{24}H_{60}O_6Si_6$	612.2926 – $[C_{24}H_{60}O_6Si_6]^+$ 305.1403 – $[C_{12}H_{29}O_3Si_3]^+$ 217.1059 – $[C_9H_{21}O_2Si_2]^+$	Carbohydrates (sugar alcohols)

Continuation Table 1

Maltose	23.61	$C_{12}H_{22}O_{11}$	69-79-4	$C_{36}H_{86}O_{11}Si_8$	243.1212 – $[C_{15}H_{19}OSi]^+$ 204.0994 – $[C_8H_{20}O_2Si_2]^+$ 191.0887 – $[C_{11}H_{15}OSi]^+$	Carbohydrates (disaccharides)
	24.19			$C_{37}H_{89}NO_{11}Si_8$	480.2422 – $[C_{23}H_{42}NO_4Si_3]^+$ 361.1666 – $[C_{15}H_{33}O_4Si_3]^+$ 319.1558 – $[C_{17}H_{27}O_2Si_2]^+$	
	24.35			$C_{36}H_{86}O_{11}Si_8$	361.1681 – $[C_{15}H_{33}O_4Si_3]^+$ 204.0995 – $[C_8H_{20}O_2Si_2]^+$ 191.0887 – $[C_{11}H_{15}OSi]^+$	
Alanine	7.00	$C_3H_7NO_2$	56-41-7	$C_9H_{23}NO_2Si_2$	218.1013 – $[C_8H_{20}NO_2Si_2]^+$ 190.1067 – $[C_7H_{20}NOSi_2]^+$ 116.0885 – $[C_5H_{14}NSi]^+$	Amino acids
Valine	8.70	$C_5H_{11}NO_2$	72-18-4	$C_{11}H_{27}NO_2Si_2$	246.1324 – $[C_{10}H_{24}NO_2Si_2]^+$ 218.1110 – $[C_9H_{22}O_2Si_2]^+$ 203.0809 – $[C_7H_{17}NO_2Si_2]^+$	Amino acids
Leucine	9.50	$C_6H_{13}NO_2$	61-90-5	$C_{12}H_{29}NO_2Si_2$	260.1489 – $[C_{11}H_{26}NO_2Si_2]^+$ 232.1535 – $[C_{10}H_{26}NOSi_2]^+$ 158.1357 – $[C_8H_{20}NSi]^+$	Amino acids
Isoleucine	9.80	$C_6H_{13}NO_2$	73-32-5	$C_{12}H_{29}NO_2Si_2$	260.1489 – $[C_{11}H_{26}NO_2Si_2]^+$ 232.1535 – $[C_{10}H_{26}NOSi_2]^+$ 158.1357 – $[C_8H_{20}NSi]^+$	Amino acids
Proline	9.87	$C_5H_9NO_2$	609-36-9	$C_{11}H_{25}NO_2Si_2$	216.1224 – $[C_9H_{22}NOSi_2]^+$ 170.0620 – $[C_7H_{12}NO_2Si]^+$ 142.1043 – $[C_7H_{16}NSi]^+$	Amino acids
Glycine	7.28	$C_2H_5NO_2$	56-40-6	$C_8H_{21}NO_2Si_2$	204.0876 – $[C_7H_{18}NO_2Si_2]^+$ 176.0914 – $[C_6H_{18}NOSi_2]^+$ 102.0735 – $[C_4H_{12}NSi]^+$	Amino acids
	9.99			$C_{11}H_{29}NO_2Si_3$	276.1248 – $[C_{10}H_{26}NO_2Si_3]^+$ 248.1303 – $[C_9H_{26}NOSi_3]^+$ 174.1125 – $[C_7H_{20}NSi_2]^+$	
Serine	9.28	$C_3H_7NO_3$	302-84-1	$C_9H_{23}NO_3Si_2$	234.0954 – $[C_8H_{20}NOSi_2]^+$ 206.0477 – $[C_9H_{12}NOSi_2]^+$ 193.0507 – $[C_9H_{13}OSi_2]^+$	Amino acids
	10.71			$C_{12}H_{31}NO_3Si_3$	218.1033 – $[C_8H_{20}NO_2Si_2]^+$ 204.1283 – $[C_8H_{22}NOSi_2]^+$ 188.0924 – $[C_7H_{18}NOSi_2]^+$	
Aspartic acid	11.25	$C_4H_7NO_4$	617-45-8	$C_{10}H_{23}NO_4Si_2$	262.0921 – $[C_9H_{20}NO_4Si_2]^+$ 172.0462 – $[C_6H_{10}NO_3Si]^+$ 160.0787 – $[C_6H_{14}NO_2Si]^+$	Amino acids
Aminomalonic acid	12.14	$C_3H_5NO_4$	1068-84-4	$C_{12}H_{29}NO_4Si_3$	320.1163 – $[C_{11}H_{26}NO_4Si_3]^+$ 292.1216 – $[C_{10}H_{26}NO_3Si_3]^+$ 218.1028 – $[C_8H_{20}NO_2Si_2]^+$	Amino acids
Ornithine	13.93	$C_5H_{12}N_2O_2$	70-26-8	$C_{14}H_{36}N_2O_2Si_3$	348.2070 – $[C_{14}H_{36}N_2O_2Si_3]^+$ 142.1039 – $[C_7H_{16}NSi]^+$ 115.0789 – $[C_5H_{13}NSi]^+$	Amino acids
	15.48			$C_{14}H_{36}N_2O_2Si_3$	348.2058 – $[C_{14}H_{36}N_2O_2Si_3]^+$ 258.1329 – $[C_{11}H_{24}NO_2Si_2]^+$ 244.1166 – $[C_{14}H_{18}NOSi]^+$	
Glutamic acid	13.98	$C_5H_9NO_4$	56-86-0	$C_{14}H_{33}NO_4Si_3$	348.1465 – $[C_{13}H_{30}NO_4Si_3]^+$ 246.1333 – $[C_{10}H_{24}NO_2Si_2]^+$ 230.1019 – $[C_9H_{20}NO_2Si_2]^+$	Amino acids
Phenylalanine	14.07	$C_9H_{11}NO_2$	63-91-2	$C_{15}H_{27}NO_2Si_2$	294.1339 – $[C_{14}H_{24}NO_2Si_2]^+$ 218.1021 – $[C_8H_{20}NO_2Si_2]^+$ 192.1194 – $[C_{11}H_{18}NSi]^+$	Amino acids
Lysine	14.99	$C_6H_{14}N_2O_2$	657-27-2	$C_{15}H_{38}N_2O_2Si_3$	362.2214 – $[C_{15}H_{38}N_2O_2Si_3]^+$ 230.1162 – $[C_{10}H_{22}NOSi_2]^+$ 156.1187 – $[C_8H_{18}NSi]^+$	Amino acids

Continuation Table 1

Tyrosine	16.90	$C_9H_{11}NO_3$	60-18-4	$C_{15}H_{27}NO_3Si_2$	310.1272 – $[C_{14}H_{24}NO_3Si_2]^+$ 208.1133 – $[C_{15}H_{14}N]^+$ 179.0877 – $[C_{10}H_{15}OSi]^+$	Amino acids
	17.42			$C_{18}H_{35}NO_3Si_3$	382.1682 – $[C_{17}H_{32}NO_3Si_3]^+$ 354.1731 – $[C_{16}H_{32}NO_2Si_3]^+$ 280.1538 – $[C_{14}H_{26}NOSi_2]^+$	
Histidine	17.21	$C_6H_9N_3O_2$	71-00-1	$C_{15}H_{33}N_3O_2Si_3$	254.1495 – $[C_{11}H_{24}N_3Si_2]^+$ 218.1029 – $[C_{12}H_{14}N_2O_2]^+$ 154.0918 – $[C_7H_{14}N_2Si]^+$	Amino acids
Urocanic acid	18.02	$C_6H_6N_2O_2$	104-98-3	$C_{12}H_{22}N_2O_2Si_2$	282.1204 – $([C_{12}H_{18}NO_2Si_2]+NH_4)^+$ 267.0975 – $([C_{11}H_{15}NO_2Si_2]+NH_4)^+$ 193.0784 – $[C_9H_{13}N_2OSi]^+$	Amino acids
Tryptophan	19.92	$C_{11}H_{12}N_2O_2$	73-22-3	$C_{17}H_{28}N_2O_2Si_2$	231.1277 – $[C_{10}H_{23}O_2Si_2]^+$ 219.1112 – $[C_{12}H_{15}N_2O_2]^+$ 202.1044 – $([C_{12}H_{12}Si]+NH_4)^+$	Amino acids
Oxalic acid	7.43	$C_2H_2O_4$	144-62-7	$C_8H_{18}O_4Si_2$	219.0480 – $[C_7H_{15}O_4Si_2]^+$ 190.0831 – $[C_7H_{18}O_2Si_2]^+$ 131.0346 – $[C_4H_{11}OSi_2]^+$	Dicarboxylic acids
Succinic acid	10.09	$C_4H_6O_4$	110-15-6	$C_{10}H_{22}O_4Si_2$	262.1050 – $[C_{10}H_{22}O_4Si_2]^+$ 247.0809 – $[C_9H_{19}O_4Si_2]^+$ 203.0590 – $[C_7H_{15}O_3Si_2]^+$	Dicarboxylic acids
Fumaric acid	10.59	$C_4H_4O_4$	110-17-8	$C_{10}H_{20}O_4Si_2$	245.0659 – $[C_9H_{17}O_4Si_2]^+$ 217.0707 – $[C_8H_{17}O_3Si_2]^+$ 143.0518 – $[C_6H_{11}O_2Si]^+$	Dicarboxylic acids
Methyl-enesuccinic acid	11.77	$C_5H_6O_4$	97-65-4	$C_{11}H_{22}O_4Si_2$	259.0835 – $[C_1oH_{19}O_4Si_2]^+$ 215.0912 – $[C_9H_{19}O_2Si_2]^+$ 185.0626 – $[C_8H_{13}O_3Si]^+$	Dicarboxylic acids
Malic acid	12.37	$C_4H_6O_5$	617-48-1	$C_{13}H_{30}O_5Si_3$	245.0660 – $[C_9H_{17}O_4Si_2]^+$ 233.1022 – $[C_9H_{21}O_3Si_2]^+$ 217.0713 – $[C_8H_{17}O_3Si_2]^+$	Dicarboxylic acids
Azelaic acid	15.97	$C_9H_{16}O_4$	123-99-9	$C_{15}H_{32}O_4Si_2$	317.1575 – $[C_{14}H_{29}O_4Si_2]^+$ 217.1069 – $[C_9H_{21}O_2Si_2]^+$ 204.1015 – $[C_8H_{20}O_2Si_2]^+$	Dicarboxylic acids
Citric acid	16.17	$C_6H_8O_7$	77-92-9	$C_{18}H_{40}O_7Si_4$	465.1600 – $[C_{17}H_{37}O_7Si_4]^+$ 347.1146 – $[C_{13}H_{27}O_5Si_3]^+$ 273.0960 – $[C_{11}H_{21}O_4Si_2]^+$	Tricarboxylic acids
Lactic acid	6.32	$C_3H_6O_3$	79-33-4	$C_9H_{22}O_3Si_2$	233.1064 – $[C_9H_{21}O_3Si_2]^+$ 219.0923 – $[C_8H_{19}O_3Si_2]^+$ 203.0604 – $[C_7H_{15}O_3Si_2]^+$	Carboxylic acids (hydroxy acids)
Glycolic acid	6.58	$C_2H_4O_3$	79-14-1	$C_8H_{20}O_3Si_2$	205.0698 – $[C_7H_{17}O_3Si_2]^+$ 177.0744 – $[C_6H_{17}O_2Si_2]^+$ 161.0308 – $[C_6H_{17}OSi_2]^+$	Carboxylic acids (hydroxy acids)
Diethyl tartrate	14.07	$C_8H_{14}O_6$	87-91-2	$C_{14}H_{30}O_6Si_2$	335.1385 – $[C_{13}H_{27}O_6Si_2]^+$ 248.1131 – $[C_{10}H_{20}O_5Si]^+$ 204.1232 – $[C_9H_{20}O_3Si]^+$	Carboxylic acids (hydroxy acids)
2-Deoxy-D-erythro-pentopyra-nose	14.07	$C_5H_{10}O_4$	113890-34-9	$C_{14}H_{34}O_4Si_3$	260.0766 – $[C_9H_{20}O_3Si_3]^+$ 245.0894 – $[C_9H_{21}O_2Si_3]^+$ 217.0760 – $[C_8H_{17}O_3Si_2]^+$	Carboxylic acids (hydroxy acids)
Pyruvic acid	6.14	$C_3H_4O_3$	127-17-3	$C_7H_{15}NO_3Si$	189.0811 – $[C_7H_{15}NO_3Si]^+$ 174.0583 – $[C_6H_{12}NO_3Si]^+$ 158.0625 – $[C_6H_{12}NO_2Si]^+$	Carboxylic acids (keto acids)
Urea	8.16	CON_2H_4	57-13-6	$C_{10}H_{28}N_2OSi_3$	261.1255 – $[C_9H_{25}N_2OSi_3]^+$ 245.0944 – $[C_8H_{21}N_2OSi_3]^+$ 130.0493 – $[C_4H_{12}NSi_2]^+$	Amino acid derivative

Continuation Table 1

	9.01			$C_7H_{20}N_2OSi_2$	204.1110 – $[C_7H_{20}N_2OSi_2]^+$ 189.0885 – $[C_6H_{17}N_2OSi_2]^+$ 171.0781 – $[C_6H_{15}N_2Si_2]^+$	
3-Amino-2-piperidone	11,98	$C_5H_{10}N_2O$	42538-31-8	$C_{11}H_{26}N_2OSi_2$	258.1551 – $[C_{11}H_{26}N_2OSi_2]^+$ 243.1327 – $[C_{10}H_{23}N_2OSi_2]^+$ 203.1135 – $[C_8H_{21}NOSi_2]^+$	Piperidines
Sulfate	7.85	SO_4^{2-}	7757-82-6	$C_6H_{18}O_4SSi_2$	227.0233 – $[C_5H_{15}O_4SSi_2]^+$ 210.9973 – $[C_4H_{11}O_4SSi_2]^+$ 138.9514 – $[CH_3O_4SSi]^+$	Non-metal oxoanionic compounds
Phosphate	9.47	PO_4^{3-}	7778-53-2	$C_9H_{27}O_4PSi_3$	314.0950 – $[C_9H_{27}O_4PSi_3]^+$ 299.0722 – $[C_8H_{24}O_4PSi_3]^+$ 283.0427 – $[C_7H_{20}O_4PSi_3]^+$	Non-metal oxoanionic compounds
Caproic acid	6.56	$C_6H_{12}O_2$	142-62-1	$C_9H_{20}O_2Si$	173.0995 – $[C_8H_{17}O_2Si]^+$ 159.0829 – $[C_7H_{15}O_2Si]^+$ 131.0492 – $[C_9H_7O]^+$	Lipids (fatty acids and conjugates)
Caprylic acid	9.41	$C_8H_{16}O_2$	124-07-2	$C_{11}H_{24}O_2Si$	216.1495 – $[C_{11}H_{24}O_2Si]^+$ 201.1293 – $[C_{10}H_{21}O_2Si]^+$ 129.0384 – $[C_5H_9O_2Si]^+$	Lipids (fatty acids and conjugates)
Pelargonic acid	10.75	$C_9H_{18}O_2$	112-05-0	$C_{12}H_{26}O_2Si$	215.1465 – $[C_{11}H_{23}O_2Si]^+$ 145.0670 – $[C_6H_{13}O_2Si]^+$ 117.0370 – $[C_4H_9O_2Si]^+$	Lipids (fatty acids and conjugates)
Capric acid	12.03	$C_{10}H_{20}O_2$	334-48-5	$C_{13}H_{28}O_2Si$	229.1604 – $[C_{12}H_{25}O_2Si]^+$ 129.0375 – $[C_5H_9O_2Si]^+$ 117.0366 – $[C_4H_9O_2Si]^+$	Lipids (fatty acids and conjugates)
Lauric acid	14.38	$C_{12}H_{24}O_2$	143-07-7	$C_{15}H_{32}O_2Si$	272.2159 – $[C_{15}H_{32}O_2Si]^+$ 257.1921 – $[C_{14}H_{29}O_2Si]^+$ 145.0665 – $[C_6H_{13}O_2Si]^+$	Lipids (fatty acids and conjugates)
Palmitic acid	18.49	$C_{16}H_{32}O_2$	57-10-3	$C_{19}H_{40}O_2Si$	328.2762 – $[C_{19}H_{40}O_2Si]^+$ 313.2541 – $[C_{18}H_{37}O_2Si]^+$ 285.2229 – $[C_{16}H_{33}O_2Si]^+$	Lipids (fatty acids and conjugates)
Pentade-canoic acid	17.53	$C_{15}H_{30}O_2$	1002-84-2	$C_{18}H_{38}O_2Si$	314.2593 – $[C_{18}H_{38}O_2Si]^+$ 299.2386 – $[C_{17}H_{35}O_2Si]^+$ 271.2087 – $[C_{15}H_{31}O_2Si]^+$	Lipids (fatty acids and conjugates)
Stearic acid	20.29	$C_{18}H_{36}O_2$	57-11-4	$C_{21}H_{44}O_2Si$	356.3080 – $[C_{21}H_{44}O_2Si]^+$ 341.2854 – $[C_{20}H_{41}O_2Si]^+$ 313.2534 – $[C_{18}H_{37}O_2Si]^+$	Lipids (fatty acids and conjugates)
Eicosapen-taenoic acid	23.44	$C_{20}H_{30}O_2$	10417-94-4	$C_{26}H_{46}O_2Si$	361.1667 – $[C_{23}H_{25}O_2Si]^+$ 169.0675 – $[C_8H_{13}O_2Si]^+$ 129.0670 – $[C_{10}H_9]^+$	Lipids (fatty acids and conjugates)
2-Hydroxy-butyric acid	7.35	$C_4H_8O_3$	600-15-7	$C_{10}H_{24}O_3Si_2$	233.1028 – $[C_9H_{21}O_3Si_2]^+$ 205.1068 – $[C_8H_{21}O_2Si_2]^+$ 190.0834 – $[C_7H_{18}O_2Si_2]^+$	Lipids (hydroxy fatty acids)
2-Ethyl-caproic acid	7.95	$C_8H_{16}O_2$	205-743-6	$C_{11}H_{24}O_2Si$	201.1300 – $[C_{10}H_{21}O_2Si]^+$ 160.0904 – $[C_7H_{16}O_2Si]^+$ 145.0673 – $[C_6H_{13}O_2Si]^+$	Lipids (ethyl fatty acids)
1-Mono-palmitin	23.07	$C_{19}H_{38}O_4$	542-44-9	$C_{25}H_{54}O_4Si_2$	459.3341 – $[C_{24}H_{51}O_4Si_2]^+$ 371.2978 – $[C_{21}H_{43}O_3Si]^+$ 313.2553 – $[C_{18}H_{37}O_2Si]^+$	Lipids (glycerides)
1-Mono-stearin	24.49	$C_{21}H_{42}O_4$	123-94-4	$C_{27}H_{58}O_4Si_2$	487.3626 – $[C_{26}H_{55}O_4Si_2]^+$ 399.3283 – $[C_{23}H_{47}O_3Si]^+$ 341.2852 – $[C_{20}H_{41}O_2Si]^+$	Lipids (glycerides)
Squalene	24.79	$C_{30}H_{50}$	111-02-4	$C_{30}H_{50}$	367.3350 – $[C_{27}H_{43}]^+$ 81.0691 – $[C_6H_9]^+$ 69.0691 – $[C_5H_9]^+$	Lipids (prenol lipids)
Borate	9.73	BO_3^{3-}	10043-35-3	$C_9H_{27}BO_3Si_3$	262.1146 – $[C_8H_{24}BO_3Si_3]^+$ 224.0792 – $[C_8H_{18}BOSi_3]^+$ 217.0791 – $[C_6H_{19}BO_2Si_3]^+$	Exogenous
Triethanol-amine	14.05	$C_6H_{15}NO_3$	102-71-6	$C_{15}H_{39}NO_3Si_3$	350.2005 – $[C_{14}H_{36}NO_3Si_3]^+$ 262.1647 – $[C_{11}H_{28}NO_2Si_2]^+$ 218.1022 – $[C_8H_{20}NO_2Si_2]^+$	Exogenous

Continuation Table 1

Pilocarpine	18.67	$C_{11}H_{16}N_2O_2$	92-13-7	$C_{11}H_{16}N_2O_2$	208.1193 – $[C_{11}H_{16}N_2O_2]^+$ 109.0750 – $[C_6H_9N_2]^+$ 95.0598 – $[C_5H_7N_2]^+$	Exogenous

As can be seen, amino acids constitute the most abundant group of identified compounds. In fact, seven essential amino acids (isoleucine, leucine, lysine, phenylalanine, tryptophan, valine and histidine), seven no essential amino acids (alanine, proline, glutamic acid, glycine, serine, tyrosine and aspartic acid), and one no proteinogenic amino acid that plays a role in the urea cycle (ornithine) have been identified in sweat. All these amino acids had previously been identified in sweat using different protocols [2,21,27]. Intermediates in amino acids formation such as aminomalonic acid (alanine precursor) and urocanic acid (glutamic acid precursor), and an amino acid derivative as urea were identified in sweat. Among them, urocanic acid and urea had previously been identified in this biofluid [2,21].

Lipids are other key group of metabolites identified in sweat, including mainly fatty acids, a few conjugates and also a triterpene as squalene, the main component of sebum [27]. Fatty acids detected in sweat were mostly unsaturated, ranging from short to long-chains: caproic acid (C6:0), caprylic acid, (C8:0), pelargonic acid (C9:0), capric acid (C10:0), lauric acid (C12:0), pentadecanoic (C15:0), palmitic (C16:0) and stearic acid (C18:0). Only one unsaturated fatty acid —eicosapentaenoic acid (C20:5)— was identified in the analytical sample, where oleic acid [28] was not detected, in spite of being the most concentrated fatty acid in humans. All these compounds had previously been found in sweat [29,30]. Concerning the detected fatty acid derivatives, glycerol ester derivatives from C16:0 and C18:0 had also previously been identified in sweat [2], as well as the derivatives ethylcaproic acid [31] and hydroxybutyric acid [32].

Other family of metabolites detected in sweat was that of short carboxylic acids, which are also representative of crucial biochemical pathways that could be related to pathologies and/or dysfunctions [33]. It is worth mentioning the presence in this group of compounds related to the Krebs' cycle as succinic, oxalic, fumaric, citric, pyruvic and malic acids, compounds that had already been detected in this analytical sample [2,21]. Identified sugars included monosaccharides, a disaccharide, sugar alcohols and sugar acids, most of them previously identified in sweat [2,13,21].

Finally, other compounds not previously detected in sweat have been identified in this study. That is the case with benzene derivatives and no metal oxoanionic acids, and some exogenous compounds. All compounds no previously identified in sweat are listed in Supplementary Table 3.

Elucidation of sweat metabolome had previously been done using two different analytical platforms (NMR and LC–MS), but not by GC–MS. Fig. 3 plots the Venn diagram that compares the metabolites identified using each platform, which are listed in Supplementary Table 4.

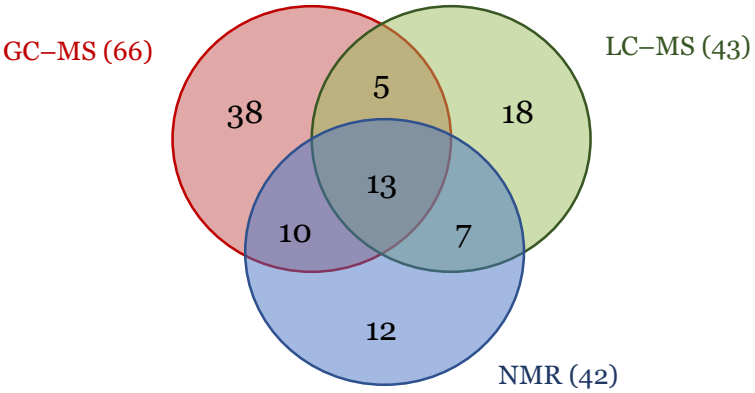

Fig. 3. Venn diagram comparing the number of compounds identified in sweat by NMR, LC–QTOF MS/MS and GC–TOF/MS.

As can be seen, a total of 103 metabolites can be detected by combination of the three platforms, being GC–MS the only one capable of detecting more than 60% of them. In fact, GC–MS is the most adequate platform to determine lipids, carbohydrates, benzene derivatives and carboxylic acids, being also useful to determine a high percent of the amino acids detected in sweat. On the other hand, NMR is capable of covering a higher number of amino acids, as well as small molecules such as ethanol, acetaldehyde or propanediol. Concerning LC–MS, this platform is also capable of covering a high percentage of amino acids, and it is the best to determine amino acid derivatives and exogenous compounds such as caffeine or theophylline.

3.3. Validation of the GC–TOF/MS method

First of all, the precision of the analytical method was evaluated by analyzing a sweat pool twice for 5 consecutive days to calculate intra-day and inter-day variability. As can be seen in Table 2, within-day variability, expressed as relative standard deviation (RSD), ranged from 0.60 to 16.99% and between-days variability, also expressed as RSD, ranged from 2.75 to 31.25%.

Table 2. Precision study for analysis of sweat metabolites estimated as percent of relative standard deviation. The study was based on 2 chromatographic injections per day on 5 consecutive days.

Compound	RT (min)	Intra-day repeatability (%)	Inter-day repeatability (%)
Pyruvic acid	6.14	4.29	5.82
Phenol	6.22	2.42	6.61
Caproic acid	6.56	16.99	17.86
Glycolic acid	6.58	8.96	12.98
Alanine	7.00	5.57	25.82
2-Hydroxybutyric acid	7.35	8.86	13.36
Oxalic acid	7.43	10.29	19.45
Sulfate	7.85	6.71	30.55
Urea	9.01	0.73	2.75
Benzoic acid	9.17	2.01	6.31
Caprylic acid	9.41	2.43	3.85
Phosphate	9.47	2.85	14.71
Glycerol	9.52	1.48	7.05
Isoleucine	9.80	1.92	12.12
Proline	9.87	4.04	24.57
Glycine	9.99	2.40	14.76
Succinic acid	10.09	0.83	7.05
Fumaric acid	10.59	2.33	7.62
Pelargonic acid	10.75	7.22	17.83
3-Amino-2-piperidone	11.98	2.87	10.07
Capric acid	12.02	5.21	7.14
Aminomalonic acid	12.14	3.29	11.91
Malic acid	12.37	2.20	6.32
Threonic acid	13.04	6.25	8.27
Ornithine	13.93	8.68	17.56
Glutamic acid	13.98	2.40	8.84

Continuation Table 2

Phenylalanine	14.07	5.86	10.33
4-Hydroxybenzoic acid	14.10	1.56	7.61
Lauric acid	14.38	4.48	11.84
Erythrulose	14.63	11.50	31.25
Lysine	14.99	6.72	7.33
Azelaic acid	15.97	13.40	9.92
Citric acid	16.17	3.17	10.92
1,5-Anhydro-D-sorbitol	16.53	3.99	10.04
Sorbose	16.72	3.77	3.13
Psicose	16.82	9.40	15.46
Tyrosine	16.90	3.26	10.67
Galactose	16.91	3.29	6.33
Glucose	16.98	3.09	5.48
Urocanic acid	18.02	13.87	18.97
Palmitic acid	18.49	1.72	6.13
Pilocarpine	18.67	7.25	12.45
Myo-Inositol	18.89	3.01	6.77
Stearic acid	20.29	0.60	4.91
1-Monopalmitin	23.07	12.39	9.75
Maltose	24.19	4.97	5.06
1-Monostearin	24.49	9.70	11.87

On the other hand, based on the identified compounds, a panel of standards was selected to characterize the proposed methodology in terms of calibration, sensitivity and recovery. The panel consisted of twelve compounds (three lipids, three amino acids, three dicarboxylic acids, one sugar, one benzene derivative and phosphate), which covered a broad range of chemical classes potentially occurring in sweat. These compounds have also been used to confirm the identified compounds by GC–TOF/MS analysis of individual standard solutions of the panel compounds subjected to the same preparation protocol as the sweat samples.

Two calibration models were built for each compound using water and sweat as matrix. In both cases, a multistandard solution was added at six concentration levels, covering in both cases a calibration range from 0.50 to 30 μg/mL. Four of the calibration points were injected in triplicate to set the confidence intervals.

Table 3. Characteristics of the developed method: linear calibration ranges, calibration equations, regression coefficients, limit of detection (LOD) and quantitation (LOQ), and estimated recovery for analysis of sweat samples spiked with panel compounds at two levels. Recovery is expressed as percent of each detected compound as compared to the spiked concentration.

Compound	Family	Calibration curve	Regression coefficient	LOD (µg/mL)	Calibration range (µg/mL)		Recovery (%)	
					LOQ	Maximum	At low concentration level[a]	At high concentration level[b]
4-Hydroxybenzoic acid	Benzene derivatives (acids)	y=(1107198 ±26908)x +788179±219589	0.9935	0.15	0.50	20.80	82.56	94.69
Galactose	Carbohydrates (monosaccharides)	y=(382616 ±5564)x +270953±53556	0.9961	0.15	0.50	20.40	101.44	99.06
Proline	Amino acids	y=(685169 ±19624)x +3147769±16013	0.9911	0.30	1.00	24.40	86.77	100.06
Glutamic acid	Amino acids	y=(221487 ±6331)x +2177124±53657	0.9919	0.30	1.00	29.00	110.60	102.65
Phenylalanine	Amino acids	y=(158972 ±6778)x +1331622±54498	0.9910	0.15	0.50	26.30	93.21	100.55
Succinic acid	Dicarboxylic acids	y=(696478 ±12786)x +1460309±10708	0.9966	0.15	0.50	21.50	99.52	98.13
Fumaric acid	Dicarboxylic acids	y=(517269± 7427)x +612626±60610	0.9977	0.15	0.50	21.20	99.49	98.62
Malic acid	Dicarboxylic acids	y=(845505 ±14714)x +1131042±117369	0.9964	0.15	0.50	21.20	97.65	98.56
Phosphate	Non-metal oxoanionic compounds	y=(492755 ±15821)x +3842416±12008	0.9939	0.15	0.50	28.10	101.95	98.79
Caprylic acid	Lipids (fatty acids and conjugates)	y=(714498 ±24940)x +371500±52906	0.9915	0.15	0.50	5.50	99.97	95.84
Lauric acid	Lipids (fatty acids and conjugates)	y=(375777 ±7827)x +401578±63873	0.9953	0.15	0.50	21.10	108.79	100.90
Palmitic acid	Lipids (fatty acids and conjugates)	y=(166917± 3322)x +160127±29388	0.9964	0.15	0.50	21.00	94.99	100.60

[a] The concentration selected for recovery estimation at low level was 5 µg/mL, except for caprylic acid, for which it was 1 µg/mL; [b] The concentration selected for recovery estimation at high level was 20 µg/mL, except for caprylic acid, for which it was 5 µg/mL.

Although the regression coefficient was similar for both calibration curves, the slope was at least 1.60 times higher in the curve obtained with sweat. Thus, the matrix effect leads to an increased sensitivity. Due to this matrix effect, the characterization of the method was done using the calibration curve in sweat and quantitation was performed by standard addition. The linear dynamic ranges and regression coefficients are shown in Table 3.

As can be seen, regression coefficients were above 0.99 for all analytes and the linear range depended on the target compound. Thus, some compounds fitted linearly up to concentrations *ca*. 29 μg/mL, while others only reached 5.50 μg/mL.

The sensitivity of the method was evaluated by estimation of the lowest limit of detection (LLOD) and that of quantitation (LLOQ), both determined by injecting in-series dilutions of the multistandard to obtain signals 3 and 10 times, respectively, the background noise (average noise value obtained for blank injections). LOD and LOQ were between 0.15–0.30 μg/mL and 0.50–1 μg/mL, respectively, for all compounds and they are also shown in Table 3.

Finally, recovery of the deproteination step was estimated by analysis of spiked sweat samples at two concentrations (5 and 20 μg/mL for all metabolites except for caprylic acid, which was spiked at 1 and 5 μg/mL because of its linear range only reach to 5.50 μg/mL), selected to cover the linear dynamic range. Recovery values, expressed as percent of the spiked concentration, are listed in Table 3. As can be seen, acceptable accuracy values were obtained for all analytes near the two extremes of the linear dynamic range, with variability values between 82 and 111%.

Although a quite straightforward sample preparation (protein precipitation followed by derivatization) was used, satisfactory validation results in terms of precision, recovery, and linearity have been achieved with some exceptions.

4. Conclusions

Sample preparation for untargeted sweat analysis has been optimized, considering both the clean-up and derivatization steps. Although sweat is gaining popularity in metabolomics studies, its metabolome has not been completely elucidated yet. Thus, the optimization of a new analytical platform as GC–MS for sweat study has revealed the

presence of metabolites that can be of interest for the study of different pathologies. Among the tested protocols, sweat deproteination with subsequent methoxymation plus silylation was the best option to obtain a representative snapshot of sweat metabolome. GC–TOF/MS appears to be the best strategy to analyze this biofluid in terms of metabolome coverage as compared to previous studies using NMR and LC–MS. It is worthy to distinguishing the ability of GC–MS to detect sugars, lipids and carboxylic acids, families that with previous platforms seem to have insignificant presence, but they constitute around 20% of the whole entities detected by GC–MS. These three groups, together with amino acids that involve 30% of the identified compounds, are the four most important families in sweat.

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Supplementary material

Supplementary Table 1. Sweat compounds identified by GC–TOF/MS in each experiment.

Compound	RT (min)	Formula	Family	Deproteination	LLE	
					Dichloro-methane	Ethyl acetate
Phenol	6.22	C_6H_6O	Benzene derivatives (alcohols)	✓	✓	✓
Benzoic acid	9.17	$C_7H_6O_2$	Benzene derivatives (acids)	✓	✓	✓
4-Hydroxybenzoic acid	14.10	$C_7H_6O_3$	Benzene derivatives (acids)	✓		
Isopropyl 4-hydroxybenzoate	14.55	$C_{10}H_{12}O_3$	Benzene derivatives (esters)	✓	✓	✓
Glycerol	9.52	$C_3H_8O_3$	Carbohydrates (sugar alcohols)	✓	✓	✓
Glyceric acid	10.30	$C_3H_6O_4$	Carbohydrates (sugar acids)	✓		
Threonic acid	13.04	$C_4H_8O_5$	Carbohydrates (sugar acids)	✓		
Erythrulose	14.63	$C_4H_8O_4$	Carbohydrates (monosaccharides)	✓		
1,5-Anhydro-D-sorbitol	16.53	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)	✓		
Sorbose	16.72	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓		
Psicose	16.82	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓		✓
Galactose	16.91	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓	✓
Glucose	16.98	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓		
Myo-Inositol	18.89	$C_6H_{12}O_6$	Carbohydrates (sugar alcohols)	✓		
Maltose	24.19	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)	✓		✓
Alanine	7.00	$C_3H_7NO_2$	Amino acids	✓	✓	
Valine	8.70	$C_5H_{11}NO_2$	Amino acids	✓	✓	✓
Leucine	9.50	$C_6H_{13}NO_2$	Amino acids	✓		
Isoleucine	9.80	$C_6H_{13}NO_2$	Amino acids	✓	✓	
Proline	9.87	$C_5H_9NO_2$	Amino acids	✓	✓	
Glycine	9.99	$C_2H_5NO_2$	Amino acids	✓	✓	
Serine	10.71	$C_3H_7NO_3$	Amino acids	✓	✓	
Aspartic acid	11.24	$C_4H_7NO_4$	Amino acids	✓		
Aminomalonic acid	12.14	$C_3H_5NO_4$	Amino acids	✓		
Ornithine	13.93	$C_5H_{12}N_2O_2$	Amino acids	✓		
Glutamic acid	13.98	$C_5H_9NO_4$	Amino acids	✓	✓	
Phenylalanine	14.07	$C_9H_{11}NO_2$	Amino acids	✓		
Lysine	14.99	$C_6H_{14}N_2O_2$	Amino acids	✓		
Tyrosine	16.90	$C_9H_{11}NO_3$	Amino acids	✓		

Continuation Supplementary Table 1

Urocanic acid	18.02	$C_6H_6N_2O_2$	Amino acids	✓		✓
Oxalic acid	7.43	$C_2H_2O_4$	Dicarboxylic acids	✓	✓	
Succinic acid	10.09	$C_4H_6O_4$	Dicarboxylic acids	✓		
Fumaric acid	10.59	$C_4H_4O_4$	Dicarboxylic acids	✓		
Methylenesuccinic acid	11,77	$C_5H_6O_4$	Dicarboxylic acids	✓		
Malic acid	12.37	$C_4H_6O_5$	Dicarboxylic acids	✓		
Azelaic acid	15.97	$C_9H_{16}O_4$	Dicarboxylic acids	✓		
Citric acid	16.17	$C_6H_8O_7$	Tricarboxylic acids	✓		
Lactic acid	6.32	$C_3H_6O_3$	Carboxylic acids (hydroxy acids)	✓	✓	✓
Glycolic acid	6.58	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)	✓	✓	
2-Deoxy-D-erythro-pentopyranose	14.07	$C_5H_{10}O_4$	Carboxylic acids (hydroxy acids)	✓		
Pyruvic acid	6.14	$C_3H_4O_3$	Carboxylic acids (keto acids)	✓	✓	
Urea	9.01	CON_2H_4	Ureas	✓	✓	✓
3-Amino-2-piperidone	11.98	$C_5H_{10}N_2O$	Piperidines	✓		
Sulfate	7.85	SO_4^{2-}	Non-metal oxoanionic compounds	✓	✓	✓
Phosphate	9.47	PO_4^{3-}	Non-metal oxoanionic compounds	✓	✓	
Caproic acid	6.56	$C_6H_{12}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Caprylic acid	9.41	$C_8H_{16}O_2$	Lipids (fatty acids and conjugates)	✓		✓
Pelargonic acid	10.75	$C_9H_{18}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Capric acid	12,03	$C_{10}H_{20}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Lauric acid	14,38	$C_{12}H_{24}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Palmitic acid	18.49	$C_{16}H_{32}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Pentadecanoic acid	17.53	$C_{15}H_{30}O_2$	Lipids (fatty acids and conjugates)	✓		
Stearic acid	20.29	$C_{18}H_{36}O_2$	Lipids (fatty acids and conjugates)	✓	✓	✓
Eicosapentaenoic acid	23.44	$C_{20}H_{30}O_2$	Lipids (fatty acids and conjugates)	✓		
2-Hydroxybutyric acid	7.35	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓		
2-Ethylcaproic acid	7.95	$C_8H_{16}O_2$	Lipids (ethyl fatty acids)	✓		✓
1-Monopalmitin	23.07	$C_{19}H_{38}O_4$	Lipids (glycerides)	✓	✓	✓
1-Monostearin	24.49	$C_{21}H_{42}O_4$	Lipids (glycerides)	✓	✓	✓
Squalene	24.79	$C_{30}H_{50}$	Lipids (prenol lipids)	✓		
Borate	9.73	BO_3^{3-}	Exogenous	✓		
Triethanolamine	14.05	$C_6H_{15}NO_3$	Exogenous	✓		✓
Pilocarpine	18.67	$C_{11}H_{16}N_2O_2$	Exogenous	✓	✓	✓

Supplementary Table 2. Sweat compounds identified after derivatization with methoxymation plus silylation or only by silylation prior to GC–TOF/MS.

Compound	RT (min)	Formula	Family	Methoxymation + silylation	Silylation
Phenol	6.22	C_6H_6O	Benzene derivatives (alcohols)	✓	✓
Benzoic acid	9.17	$C_7H_6O_2$	Benzene derivatives (acids)	✓	✓
4-Hydroxybenzoic acid	14.10	$C_7H_6O_3$	Benzene derivatives (acids)	✓	
Isopropyl 4-hydroxybenzoate	14.54	$C_{10}H_{12}O_3$	Benzene derivatives (esters)	✓	✓
Glycerol	9.52	$C_3H_8O_3$	Carbohydrates (sugar alcohols)	✓	✓
Glyceric acid	10.30	$C_3H_6O_4$	Carbohydrates (sugar acids)	✓	✓
Threonic acid	13.04	$C_4H_8O_5$	Carbohydrates (sugar acids)	✓	✓
Erythrulose	14.63	$C_4H_8O_4$	Carbohydrates (monosaccharides)	✓	✓
1,5-Anhydro-D-sorbitol	16.53	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)	✓	✓
Sorbose	16.72	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	
Psicose	16.82	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓
Galactose	16.91	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓
Methyl β-D-galactopyranoside	16.95	$C_7H_{14}O_6$	Carbohydrates (monosaccharides)		✓
Glucose	16.98	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)	✓	✓
Myo-Inositol	18.89	$C_6H_{12}O_6$	Carbohydrates (sugar alcohols)	✓	✓
Maltose	24.19	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)	✓	✓
Alanine	7.00	$C_3H_7NO_2$	Amino acids	✓	✓
Valine	8.70	$C_5H_{11}NO_2$	Amino acids	✓	✓
Leucine	9.50	$C_6H_{13}NO_2$	Amino acids	✓	✓
Isoleucine	9.80	$C_6H_{13}NO_2$	Amino acids	✓	✓

Continuation Supplementary Table 2

Proline	9.87	$C_5H_9NO_2$	Amino acids	✓	✓
Glycine	9.99	$C_2H_5NO_2$	Amino acids	✓	✓
Serine	10.71	$C_3H_7NO_3$	Amino acids	✓	✓
Aspartic acid	11.25	$C_4H_7NO_4$	Amino acids	✓	✓
Aminomalonic acid	12.14	$C_3H_5NO_4$	Amino acids	✓	✓
Ornithine	13.93	$C_5H_{12}N_2O_2$	Amino acids	✓	✓
Glutamic acid	13.98	$C_5H_9NO_4$	Amino acids	✓	✓
Phenylalanine	14.07	$C_9H_{11}NO_2$	Amino acids	✓	
Lysine	14.99	$C_6H_{14}N_2O_2$	Amino acids	✓	✓
Tyrosine	16.90	$C_9H_{11}NO_3$	Amino acids	✓	✓
Histidine	17.21	$C_6H_9N_3O_2$	Amino acids		✓
Urocanic acid	18.02	$C_6H_6N_2O_2$	Amino acids	✓	✓
Tryptophan	19.92	$C_{11}H_{12}N_2O_2$	Amino acids		✓
Oxalic acid	7.43	$C_2H_2O_4$	Dicarboxylic acids	✓	✓
Succinic acid	10.09	$C_4H_6O_4$	Dicarboxylic acids	✓	✓
Fumaric acid	10.59	$C_4H_4O_4$	Dicarboxylic acids	✓	✓
Methylenesuccinic acid	11,77	$C_5H_6O_4$	Dicarboxylic acids	✓	✓
Malic acid	12.37	$C_4H_6O_5$	Dicarboxylic acids	✓	✓
Azelaic acid	15.97	$C_9H_{16}O_4$	Dicarboxylic acids	✓	✓
Citric acid	16.17	$C_6H_8O_7$	Tricarboxylic acids	✓	✓
Lactic acid	6.32	$C_3H_6O_3$	Carboxylic acids (hydroxy acids)	✓	✓
Glycolic acid	6.58	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)	✓	✓
Diethyl tartrate	14.07	$C_8H_{14}O_6$	Carboxylic acids (hydroxy acids)		✓

Continuation Supplementary Table 2

2-Deoxy-D-erythro-pentopyranose	14.07	$C_5H_{10}O_4$	Carboxylic acids (hydroxy acids)	✓	✓
Pyruvic acid	6.14	$C_3H_4O_3$	Carboxylic acids (keto acids)	✓	
Urea	9.01	CON_2H_4	Ureas	✓	✓
3-Amino-2-piperidone	11.98	$C_5H_{10}N_2O$	Piperidines	✓	✓
Sulfate	7.85	SO_4^{2-}	Non-metal oxoanionic compounds	✓	✓
Phosphate	9.47	PO_4^{3-}	Non-metal oxoanionic compounds	✓	✓
Caproic acid	6.56	$C_6H_{12}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Caprylic acid	9.41	$C_8H_{16}O_2$	Lipids (fatty acids and conjugates)	✓	
Pelargonic acid	10.75	$C_9H_{18}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Capric acid	12.03	$C_{10}H_{20}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Lauric acid	14,38	$C_{12}H_{24}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Palmitic acid	18.49	$C_{16}H_{32}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Pentadecanoic acid	17.53	$C_{15}H_{30}O_2$	Lipids (fatty acids and conjugates)	✓	
Stearic acid	20.29	$C_{18}H_{36}O_2$	Lipids (fatty acids and conjugates)	✓	✓
Eicosapentaenoic acid	23.44	$C_{20}H_{30}O_2$	Lipids (fatty acids and conjugates)	✓	✓
2-Hydroxybutyric acid	7.35	$C_4H_8O_3$	Lipids (hydroxy fatty acids)	✓	✓
2-Ethylcaproic acid	7.95	$C_8H_{16}O_2$	Lipids (ethyl fatty acids)	✓	✓
1-Monopalmitin	23.07	$C_{19}H_{38}O_4$	Lipids (glycerides)	✓	✓
1-Monostearin	24.49	$C_{21}H_{42}O_4$	Lipids (glycerides)	✓	✓
Squalene	24.79	$C_{30}H_{50}$	Lipids (prenol lipids)	✓	
Borate	9.73	BO_3^{3-}	Exogenous	✓	✓
Triethanolamine	14.05	$C_6H_{15}NO_3$	Exogenous	✓	
Pilocarpine	18.67	$C_{11}H_{16}N_2O_2$	Exogenous	✓	✓

Supplementary Table 3. Compounds no previously identified in sweat.*

Compound	RT (min)	Formula	Family
Phenol	6.22	C_6H_6O	Benzene derivatives (alcohols)
Benzoic acid	9.17	$C_7H_6O_2$	Benzene derivatives (acids)
4-Hydroxybenzoic acid	14.10	$C_7H_6O_3$	Benzene derivatives (acids)
Isopropyl 4-hydroxybenzoate	14.54	$C_{10}H_{12}O_3$	Benzene derivatives (esters)
Glyceric acid	10.30	$C_3H_6O_4$	Carbohydrates (sugar acids)
Threonic acid	13.04	$C_4H_8O_5$	Carbohydrates (sugar acids)
Erythrulose	14.63	$C_4H_8O_4$	Carbohydrates (monosaccharides)
1,5-Anhydro-D-sorbitol	16.53	$C_6H_{12}O_5$	Carbohydrates (monosaccharides)
Sorbose	16.72	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)
Psicose	16.82	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)
Galactose	16.91	$C_6H_{12}O_6$	Carbohydrates (monosaccharides)
Methyl β-D-galactopyranoside	16.95	$C_7H_{14}O_6$	Carbohydrates (monosaccharides)
Maltose	24.19	$C_{12}H_{22}O_{11}$	Carbohydrates (disaccharides)
Aminomalonic acid	12.14	$C_3H_5NO_4$	Amino acids
Methylenesuccinic acid	11,77	$C_5H_6O_4$	Dicarboxylic acids
Glycolic acid	6.58	$C_2H_4O_3$	Carboxylic acids (hydroxy acids)
Diethyl tartrate	14.07	$C_8H_{14}O_6$	Carboxylic acids (hydroxy acids)
2-Deoxy-D-erythro-pentopyranose	14.07	$C_5H_{10}O_4$	Carboxylic acids (hydroxy acids)
3-Amino-2-piperidone	11.98	$C_5H_{10}N_2O$	Piperidines
Sulfate	7.85	$S\ O_4^{2-}$	No metal oxoanionic compounds
Phosphate	9.47	PO_4^{3-}	No metal oxoanionic compounds
Borate	9.73	BO_3^{3-}	Exogenous
Triethanolamine	14.05	$C_6H_{15}NO_3$	Exogenous

*As far as the authors know.

Supplementary Table 4. Sweat compounds identified by NMR, LC–QTOF MS/MS and GC–TOF/MS.

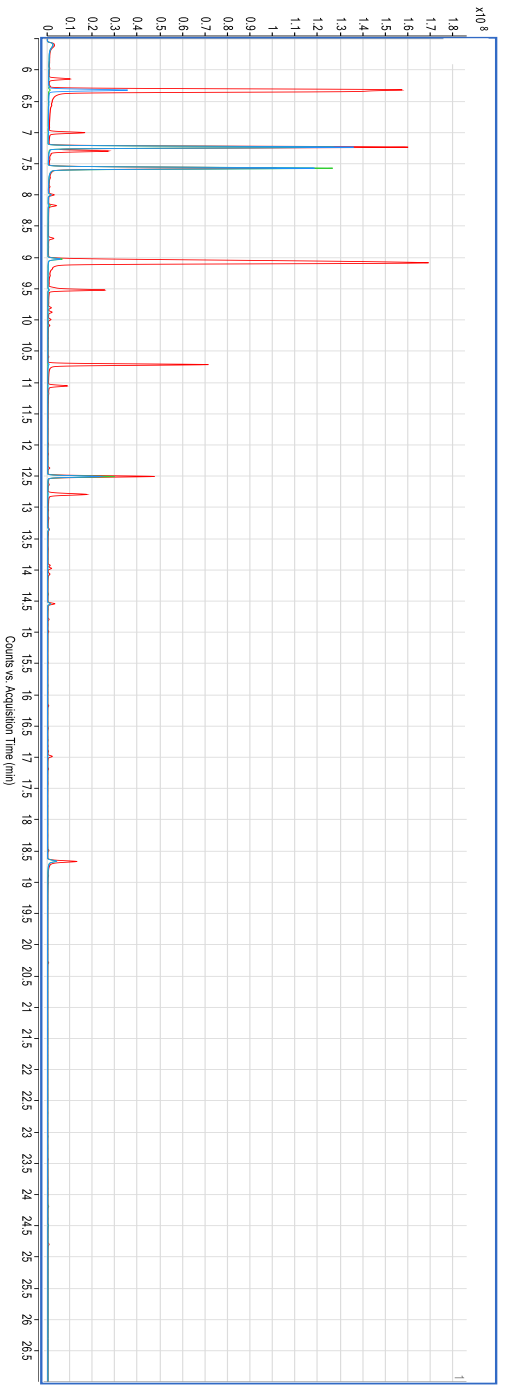
Compound	Family	NMR	LC–MS	GC–MS
Benzoic acid	Benzene derivatives			✓
4-Hydroxybenzoic acid	Benzene derivatives			✓
Isopropyl 4-hydroxybenzoate	Benzene derivatives			✓
Phenol	Benzene derivatives			✓
1,5-Anhydro-D-sorbitol	Carbohydrates			✓
Erythrulose	Carbohydrates			✓
Galactose	Carbohydrates			✓
Glucose	Carbohydrates	✓		✓
Glyceric acid	Carbohydrates			✓
Glycerol	Carbohydrates	✓		✓
Inositol	Carbohydrates	✓		✓
Maltose	Carbohydrates			✓
Maltotriose	Carbohydrates		✓	
Methyl β-D-galactopyranoside	Carbohydrates			✓
Psicose	Carbohydrates			✓
Sorbose	Carbohydrates			✓
Sucrose	Carbohydrates	✓		
Tetrasaccharide	Carbohydrates		✓	
Threonic acid	Carbohydrates			✓
Alanine	Amino acids	✓		✓
Aminomalonic acid	Amino acids			✓
Arginine	Amino acids	✓	✓	
Aspartic acid	Amino acids	✓	✓	✓
Citrulline	Amino acids	✓	✓	
Cysteine	Amino acids	✓		
Glutamic acid	Amino acids	✓	✓	✓
Glutamine	Amino acids	✓	✓	
Glycine	Amino acids	✓		✓
Histidine	Amino acids	✓	✓	✓
Isoleucine	Amino acids	✓	✓	✓
Leucine	Amino acids	✓	✓	✓
Lysine	Amino acids	✓		✓

Continuation Supplementary Table 4

Methionine	Amino acids	✓	✓	
Ornithine	Amino acids	✓	✓	✓
Phenylalanine	Amino acids	✓	✓	✓
Proline	Amino acids	✓	✓	✓
Serine	Amino acids	✓	✓	✓
Threonine	Amino acids	✓	✓	
Tryptophan	Amino acids	✓	✓	✓
Tyrosine	Amino acids	✓	✓	✓
Valine	Amino acids	✓	✓	✓
Carnitine	Amino acid derivatives		✓	
N- acetyl - aspartate	Amino acid derivatives	✓		
Prolylhydroxyproline	Amino acid derivatives		✓	
Pyroglutamic acid	Amino acid derivatives	✓	✓	
Taurine	Amino acid derivatives		✓	
Tyramine	Amino acid derivatives		✓	
Urea	Amino acid derivatives	✓		✓
Urocanic acid	Amino acid derivatives	✓	✓	✓
2-Deoxy-D-erythro-pentopyranose	Carboxylic acids			✓
Azelaic acid	Carboxylic acids		✓	✓
Cis-aconitate	Carboxylic acids	✓		
Citric acid	Carboxylic acids			✓
Diethyl tartrate	Carboxylic acids			✓
Formate	Carboxylic acids	✓		
Fumaric acid	Carboxylic acids	✓		✓
Glycolic acid	Carboxylic acids			✓
Lactic acid	Carboxylic acids	✓		✓
Malic acid	Carboxylic acids			✓
Methylenesuccinic acid	Carboxylic acids			✓
Oxalic acid	Carboxylic acids			✓
Pyruvic acid	Carboxylic acids	✓		✓
Sebacic acid	Carboxylic acids		✓	
Suberic acid	Carboxylic acids		✓	
Succinic acid	Carboxylic acids		✓	✓
Acetoacetate	Carboxylic acid derivatives	✓		
Choline	Nutrients	✓	✓	

Continuation Supplementary Table 4

Coumaric acid	Nutrients		✓	
Inosine	Purine derivatives		✓	
Uric acid	Purine derivatives		✓	
3-Amino-2-piperidone	Piperidines			✓
Phosphate	No metal oxoanionic compounds			✓
Sulfate	No metal oxoanionic compounds			✓
1-Monopalmitin	Lipids		✓	✓
1-Monostearin	Lipids		✓	✓
2-Ethylcaproic acid	Lipids			✓
2-Hydroxybutyric acid	Lipids			✓
C16 sphinganine	Lipids		✓	
Capric acid	Lipids			✓
Caproic acid	Lipids			✓
Caprylic acid	Lipids			✓
Eicosapentaenoic acid	Lipids			✓
Lauric acid	Lipids			✓
MG(22:2)	Lipids		✓	
Palmitic acid	Lipids			✓
Pelargonic acid	Lipids			✓
Pentadecanoic acid	Lipids			✓
Squalene	Lipids			✓
Stearic acid	Lipids			✓
Dimethylamine	Amines	✓		
Methylamine	Amines	✓		
Ammonium cation	Ammonium	✓		
1,2-Propanediol	Exogenous	✓		
Acetaldehyde	Exogenous	✓		
Borate	Exogenous			✓
Caffeine	Exogenous		✓	
Ethanol	Exogenous	✓		
Methylparaben	Exogenous		✓	
Pilocarpic acid	Exogenous		✓	
Pilocarpine	Exogenous		✓	✓
Propylparaben	Exogenous		✓	
Theophylline	Exogenous		✓	
Triethanolamine	Exogenous			✓



Supplementary Fig. 1. Base peak chromatograms (BPCs) provided by analysis of sweat extracts obtained with ethyl acetate (green), dichloromethane (blue) or by deproteination with methanol and acetonitrile (red).

Chapter XI

Recent advances in human sweat metabolomics for lung cancer screening

Recent advances in human sweat metabolomics for lung cancer screening

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Abstract

Introduction Lung cancer is the leading cause of cancer related mortality owing to the advanced stage it is usually detected because the available diagnostic tests are expensive and invasive; therefore, they cannot be used for general screening.

Objectives To increase robustness of previous biomarker panels — based on metabolites in sweat samples— proposed by the authors, new samples were collected within different intervals (4 months and two years), analyzed at different times (2012 and 2014, respectively) by different analysts to discriminate lung cancer patients and smokers at risk factor.

Methods Sweat analysis was carried out by LC–MS/MS with minimum sample preparation and the generated analytical data were then integrated to minimize variability in statistical analysis.

Results Panels with capability to discriminate lung cancer patients from smokers at risk factor were obtained taken into account the variability between both cohorts as a consequence of the different intervals for samples collection, the times at which the analyses were carried out and the influence of the analyst. Two panels of metabolites using the PanelomiX tool allow reducing false negatives (95% specificity) and false positives (95% sensitivity). The first panel (96.9% specificity and 83.8% sensitivity) is composed by monoglyceride MG(22:2), muconic, suberic and urocanic acids, and a tetrahexose; the second panel (81.2% specificity and 97.3% sensitivity) is composed by the monoglyceride MG(22:2), muconic, nonanedioic and urocanic acids, and a tetrahexose.

Conclusion The study has allowed obtaining a prediction model more robust than that obtained in the previous study from the authors.

Keywords: lung cancer, human sweat, biomarker panel, metabolomics, liquid chromatography, mass spectrometry.

1. Introduction

Lung cancer is the leading cause of cancer deaths in the United Kingdom (Cancer Research UK 2012), Europe (Ferlay *et al.*, 2008) and the United States (Jemal *et al.*, 2010). According to the American Cancer Society, lung cancer causes more deaths than the sum of the three most common cancers (colon, breast and prostate) (World Health Organization, 2012). Despite the advances in diagnostic, surgery, and chemotherapy, the last 30 years has seen little improvement in the overall five-year survival rate for lung cancer, with only 15% of patients living for at least five years after initial diagnosis (Jemal *et al.*, 2010). These relatively poor survival rates are mainly the result of the late detection of a malignancy for which available diagnostic tests are invasive and cannot be used for general screening.

Presently, no invasive techniques for cancer screening are the subject of wide clinical research. The current techniques to diagnostic lung cancer are mainly based on chest X-rays and sputum cytology, which have not contributed to reduce cancer mortality (Yu *et al.*, 2008). Recently, the use of techniques such as low-dose computed tomography has decreased mortality rate around 20% in comparison with chest X-ray radiography (Aberle *et al.*, 2011). Nevertheless, the main limitation of the present methods to diagnose lung cancer is ascribed to the high number of abnormalities potentially mimicking lung cancer (false positives) and the difficulty to apply diagnostic tools to the target population because of the high costs. This makes the 5 years survival rate very low owing to the advanced stage at which lung cancer is most times diagnosed.

The above limitations have led to active research in omics disciplines with two main purposes: to understand the development and biology of lung cancer and to identify potential biomarkers to detect initial stages in the development of the disease (Khadir and Tiss, 2013). Metabolomics has developed promising tools for disease diagnosis by biomarker discovery and characterization of biological pathways. Recent research has focused on metabolomics profiling for biomarkers discovery using different biological samples (tissue, urine, exhaled breath condensate or plasma) (Armitage and Barbas, 2014).

The low complexity of sweat matrix is an advantage to search for biomarkers in the study of diseases because other pathological conditions could interfere less than in more complex samples. Furthermore, sweat can be obtained easily in a no invasive manner (Calderón-Santiago *et al.*, 2014). Several studies have demonstrated the suitability of sweat

profiling for monitoring human health. For example, sweat has been used for over 50 years in the diagnostics of cystic fibrosis (Mishra *et al.*, 2007; Seia *et al.*, 2009). Furthermore, a recent study used human sweat as clinical sample to develop a screening tool for lung cancer (Calderón-Santiago *et al.*, 2015). The prediction model based on a panel of metabolites that included amino acids, sugars, and some lipids was built to discriminate patients with lung cancer from a risk factor control group. This panel, composed by five metabolites, provided 80% specificity and 79% sensitivity. These preliminary results, obtained with a unique cohort, emphasized the necessity of new studies to validate the obtained results and reduce the proportion of individuals that would be subjected to confirmatory tests.

The aim of the present study was to combine the data generated by analysis of sweat from individuals pertaining to two independent cohorts that were collected within different time intervals and analyzed at different times by different analysts. A crucial task was to minimize the variability between the two cohorts to make possible the integration of the data provided by analysis of both batches. Statistical analysis of the integrated data set was targeted at finding robust panels with capability to discriminate patients with lung cancer from smokers as risk factor individuals. These panels attempt to reduce the probability of false negatives and false positives, which is of crucial interest for diagnostic purposes.

2. Materials and methods

2.1. Chemicals and reagents

MS-grade formic acid (FA) and acetonitrile (ACN) from Scharlab (Barcelona, Spain) were used to prepare the chromatographic mobile phases for LC–MS/MS analysis. FA was also used for sample preparation. Deionized water (18 mΩ • cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the aqueous chromatographic phases and for sample preparation.

2.2. Apparatus and instruments

A vortex shaker from IKA (Wilmington, NC, USA) was used to shake and

homogenize the analytical samples, and an Agilent 1200 Series LC system (consisting of a binary pump, a vacuum degasser, an autosampler, and a thermostated column compartment) coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with a dual electrospray ionization (ESI) source (Santa Clara, CA, USA) was used to analyze them. The chromatographic eluate was monitored in high-resolution mode.

2.3. Cohorts selected for the study

A first cohort of thirty-five patients recruited by the Respiratory Medicine Department of the Reina Sofía Hospital (Córdoba, Spain) from March to August 2012 and a second cohort composed by thirty-four patients recruited by the same Department from November 2012 to October 2014 constituted the target of the study. Sixteen patients of the first cohort and twenty-one patients of the second were diagnosed with lung cancer after clinical tests based on bronchoscopy, fine needle biopsy, or thorascopy. Nineteen patients of the first cohort and thirteen patients of the second were classified as risk factor individuals owing to smoking habit.

In both cohorts the existence of other severe pulmonary diseases (interstitial pneumonitis, pneumonia, tuberculosis, etc.) was excluded by bronchoscopy and/or computed tomography scanning. The exclusion criteria were (1) coexistence of extrapulmonary neoplasia during the last 5 years or chemotherapy for other different neoplasias, and (2) severe organ disease with negative influence on prognostic or with influence on the application of the target protocol, including congestive heart failure, chronic liver disease (functional stage MELD >12), and chronic kidney disease stage 5 with substitutive treatment (hemodialysis or peritoneal dialysis).

The study was approved by the ethical committee of the Reina Sofia University Hospital. The individuals selected for this study were previously informed to obtain complete consent. All steps from sweat extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki of 2004. It should be emphasized that sweat collection was performed before finishing the clinical study, which means that patients were not subjected to cytostatic treatment. Therefore, medication was exclusively restricted to control the symptoms by analgesics (paracetamol or ibuprofen), cough suppressants (codeine), and similar drugs.

Apart from that, both control individuals and patients were taking common treatments related to diseases associated to the age interval (statins, antihypertensives, analgesics, etc.).

The main characteristics —age, sex, body mass index (BMI), and smoked pack-years— of the cohorts are shown in Table 1. Dependence of these characteristics among them and the risk factor group was checked by $\chi 2$ test for sex, and analysis of variance (ANOVA) with 95% confidence level for the rest of parameters but age was evaluated by Kruskal-Wallis test as this parameter did not fit a normal distribution according to the Skewness and Kurtosis criteria for normality (*p*-values are also shown in Table 1).

Table 1. Main characteristics of the two cohorts under study including age, sex, body mass index (BMI), and smoked pack-years.

Characteristics	Cohort 1 (n = 35)			Cohort 2 (n = 34)		
	Lung cancer patients (n=16)	Risk factor individuals (n=19)	*p*-Value[b]	Lung cancer patients (n=21)	Risk factor individuals (n=13)	*p*-Value[b]
Age, (year)	65 (46–87)	56 (38–77)	0.024	62 (49–75)	60 (46–73)	0.384
Sex, (male %)	81	89	0.489	81	85	0.785
Pack-years[a]	52 (20–100)	36 (10–80)	0.030	49 (0–100)	39 (15–70)	0.209
BMI, (kg/m^2)	27.0 (17.6–33.6)	28.2 (23.5–36.3)	0.407	26.0 (19.3–35.5)	29.0 (21.3–36.9)	0.126

All data are expressed as percent or mean (range); [a] Cigarettes consumption is considered as pack-years (average cigarettes smoked per day/20)×(years of smoking); [b] *p*-Values were obtained by Chi Squares test for the sex and by analysis of variance (ANOVA) for the rest of parameters —the no parametric version (Kruskal-Wallis) was applied in the case of age, since this factor does not fit normal distribution.

The first cohort revealed high significance for age (*p*-value 0.024) —lung cancer patients were older than risk factor individuals—, and for pack-years parameter (*p*-value 0.030). On the other hand, no significant factors were found in the second cohort.

The present accepted guidelines for pathological and staging diagnosis of lung cancer were used (Sánchez de Cos *et al.*, 2011). The most frequent diagnostic was squamous cell carcinoma (n=18 patients, 48.7%), followed by adenocarcinome (11 patients, 29.7%), bronchogenic carcinoma (2 patients, 5.4%), and synchronous bronchial cancer (1 patient, 2.7%). Five patients were diagnosed with no small cell lung cancer without histological classification. Concerning stages, nine cases were diagnosed as stage IA, four as stage IB, two as stage IIB, twelve as stage IIIA, four as stage IIIB, and six as stage IV. The number of patients for each diagnostic and stage did not enable inclusion of this variability source in the study.

2.4. Sweat sampling and storage

The Macroduct® Sweat Analysis System (Wescor, Utah, USA) used for sampling consists of a Webster sweat inducer and a Macroduct sweat collector (US Patent 4,542,751). Pilogel®discs (US Patent 4,383,529) (Wescor, Utah, USA), which consist of a gel reservoir of pilocarpinium ions, were used in the iontophoretic stimulation of sweat excretion. The sweat inducer provides a current intensity of 1.5 mA for 5 min through two Pilogel discs as electrodes located on the forearm. Previously, the skin was cleaned with ethanol and then with distilled water. The Macroduct device collected sweat for 15 min, which was transferred to plastic microEppendorf tubes and stored at −80 ºC until analysis. A total sweat volume of *ca*. 50 μL was collected per individual.

2.5. Sample treatment

Sweat samples (10 μL) were 1:2 (v/v) diluted with 0.1% FA in water. The mixture was vortexed for homogenezation for 1 min and placed in the LC autosampler for subsequent analysis. All samples were prepared in duplicate.

2.6 LC–QTOF MS/MS analysis

Chromatographic separation was performed using a C18 reverse-phase analytical column (Mediterranean, 50 mm×0.46 mm i.d., 3 μm particle size) from Teknokroma (Barcelona, Spain), which was thermostated at 25 °C. The mobile phases were water (phase A) and ACN (phase B) both with 0.1 % FA as ionization agent. The LC pump was

programmed with a flow rate of 0.8 mL/min with the following elution gradient: 3 % phase B as initial mobile phase was kept constant from min 0 to 1; then, the percentage of phase B was increased from 3 to 80% min 1 to 10.5, and finally from 80 to 100% of phase B from min 10.5 to 11.5. A post-time of 5 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 3 μL and the injector needle was washed ten times between injections with 70% methanol. Furthermore, the needle seat back was flushed for 15 s at a flow rate of 4 mL/min with 70% methanol to avoid cross-contamination. The parameters of the electrospray ionization source, operating in negative and positive ionization modes, were as follows: the capillary and fragmentor voltage were set at ±3.5 kV and 175 V, respectively; N2 in the nebulizer flowed at 40 psi; the flow rate and temperature of the N2 as drying gas were 8 L/min and 350 °C, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. MS and MS/MS data were collected in both polarities using the centroid mode at a rate of 1 spectrum/s in the extended dynamic range mode (2 GHz). Accurate mass spectra in MS scan and MS/MS mode were acquired in the *m/z* range 60–1100. The instrument gave typical resolution 15,000 FWHM (full width at half maximum) at *m/z* 118.0862 and 30,000 FWHM at *m/z* 922.0098. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by using the signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H,1H,3Htetrafluoro-propoxy) phosphazine or HP-921] in the positive ionization mode, while in negative ionization mode ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.0007 (formate adduct of HP-921) were used. The samples were injected in auto MS/MS acquisition mode with a preferred list of precursor ions to obtain the product ion information of metabolites with significant difference between the groups under study (two replicates per sample). Supplementary Table 1 shows the list of metabolites with significant difference between the groups under study including the retention time and *m/z* values for precursor and product ions as well as the error in precursor ion detection expressed as ppm. The number of precursors selected per cycle was set at 2, with an exclusion window of 0.3 min after two consecutive selections of the same precursor. The collision energy 20 eV was used to obtain the fragmentation information for each entity.

2.7. Data processing and statistical analysis

The MassHunter Workstation software package (Qualitative Analysis (version

B.07.00) and Profinder (version B.06.00), Agilent Technologies, Santa Clara, CA, USA) was used for processing the raw data files obtained by LC–QTOF in auto MS/MS acquisition mode. The recursive feature extraction algorithm in the software MassHunter Profinder was used to extract and align potential molecular features in all injections. This algorithm initially deconvolutes chromatograms and aligns features across the selected sample files in terms of mass and retention time; then, it uses the mass and retention time of each feature for recursive targeted feature extraction. This two-step procedure reduces the number of both false negatives and false positives in feature extraction. The target parameters for feature extraction included a threshold of 500 counts and a maximum charge state of one. In addition, the isotopic distribution for a valid feature had to be defined by two or more ions, with a peak spacing tolerance of 0.0025 *m/z*, plus 10.0 ppm. Adduct formation in the positive (+H, +Na) and negative ionization mode (–H, +HCOO) was also used to identify features of the same molecule. The features were aligned by using a tolerance window of 0.50 min and a mass accuracy of 10 ppm for retention times and *m/z* values across all data files, respectively. Identification of the metabolites detected in the two groups under study (Supplementary Table 1) was done using MS and MS/MS information and searching in the METLIN MS and MS/MS database (URL: http://metlin.scripps.edu) and Human Metabolome Database (URL: http://www.hmdb.ca/) (version 3.5). The average area of the two injections of these metabolites was calculated for each of them. MetaboAnalyst (www.metaboanalyst.ca) (version 3.0) was used for metabolomic data analysis, visualization and interpretation, and also for normalization by sum (the quantitative response of a potential metabolite is scaled by the sum of the quantitative responses of all potential metabolites detected in the samples), log transformation and scaling of the data set. The scaling procedure was carried out with the weighted mean and standard deviation. Both parameters were calculated taking into account the two classes under study: risk factor controls and lung cancer patients. The predictive capability of each metabolite by Random Forest Analysis was also determined by MetaboAnalyst. Finally, PanelomiX toolbox, supported on the iterative combination of biomarkers and thresholds, was used to combine iteratively biomarkers by selecting thresholds that provide optimal classification performance (Robin *et al.*, 2013). Cross-validation was performed by including the scaling process in it. For this purpose, 10% of the samples were excluded from the complete set. After that, data treatment was applied from scaling to panels generation. It is worth mentioning that scaling was carried out as previously described using the weighted mean and standard deviation for each

independent class. Then, the resulting panel was validated using the excluded samples. Previously, the excluded samples were scaled with the mean and standard deviation values obtained from the corresponding batch used to generate the panels. The process was repeated 10 times, which means that all samples were used for validation.

3. Results and discussion

3.1. Inter- and intra-batch variability

As mentioned before, the first cohort was sampled from March 2012 to August 2012 and the samples were analyzed in 2012 by analyst 1 (Calderón-Santiago *et al.*, 2015). Two years later, the second cohort was sampled from November 2012 to October 2014 and analyzed by analyst 2. In the first study, the relative standard deviation (RSD) for MS signals of metabolites identified in sweat in both positive and negative ionization modes was below 20%; indicative of a representative data set. In the present research, the data were generated from two independent batches. Supplementary Fig. 1 shows the bars diagram comparing the mean quantitative response and RSD obtained for each metabolite identified in the samples pertaining to each cohort and, thus, analyzed by different analysts. The sensitivity obtained from the data provided by analysis of the second batch was clearly better, except for trisaccharide phosphate, trihexose and tetrahexose, eluted in the first part of the chromatogram, and sharing a similar chemical structure. On the other hand, most of the compounds detected in positive ionization mode presented lower RSD for the analyst 2, while compounds detected in negative ionization mode presented lower variability for the analyst 1. In an attempt to evaluate the intra-batch variability, the RSD for the sum of the quantitative responses of all compounds detected in the duplicate analysis of each individual was calculated. The sum of all compounds provides evidences of the whole variability between replicates and also establishes the suitability of this parameter for normalization. Inter-replicate variability was between 0.002 and 18.4% for the first batch, and from 0.40 to 10.4% for the second batch. The mean variability, also expressed as RSD, was quite similar in both batches (4.4 and 5.8% for the first and second batch, respectively). Despite both batches provided similar RSD, differences for specific compounds were identified. Thus, it was mandatory to identify and minimize variability sources that could be associated to the experimental plan (sampling period, analysis time and analyst). Several

research groups have emphasized the capability of autoscaling and range scaling normalizations to preprocess data from metabolomics experiments without modification of the biological variability (Van den Berg *et al.*, 2006). Taking into account differences in sensitivity between both batches, the variability in terms of RSD for each identified metabolite, and also the differences in the number of samples between batches, a scaling procedure similar to autoscaling but based on weighted average and weighted standard deviation was used as preprocessing strategy (since now called weighted scaling). Thus, the weighted scaling consists of the independent calculation of the mean and standard deviation for controls and lung cancer patients, and the use of the average of means and standard deviations for the scaling procedure. This strategy, applied after normalization by sum and logarithmic transformation, allows rendering each variable on the same scale. All variables in the data set presented a normal distribution with this approach. PCA can detect systematic no biologically related or undesired bias defined as batch effects, which are recognized as frequent misguide of biological interpretations. To detect variability sources, an unsupervised principal component analysis (PCA) was firstly carried out using the combined data set that was subjected to normalization by sum, log transformation and weighted scaling. The PCA scores plot in Fig. 1A shows that variability among batches is high enough for a clear discrimination between the two cohorts by using the two first components: principal component 1 (PC1) explains 33.8%, and principal component 2 (PC2) explains 21%.

No discrimination was observed under these conditions according to the diagnostic between lung cancer patients and risk factor individuals (Fig. 1B). As the main variability source between batches was ascribed to sensitivity differences between both batches, normalization by sum, log transformation and weighted scaling were separately applied to the two data sets obtained by analysis of both batches, and then, the two resulting data sets were combined. The PCA scores plot obtained in this case (Fig. 1C) did not provide discrimination between cohorts 1 and 2, and what is more relevant, allowed detecting a slight discrimination between lung cancer and control individuals according to the second component (Fig. 1D). In this case the variability explained by PC1 and PC2 was similar to that observed in the previous PCA. With these premises, the separated application of normalization, transformation and weighted scaling prior to combination of both data sets was crucial to remove the contribution of external variability sources associated to each cohort.

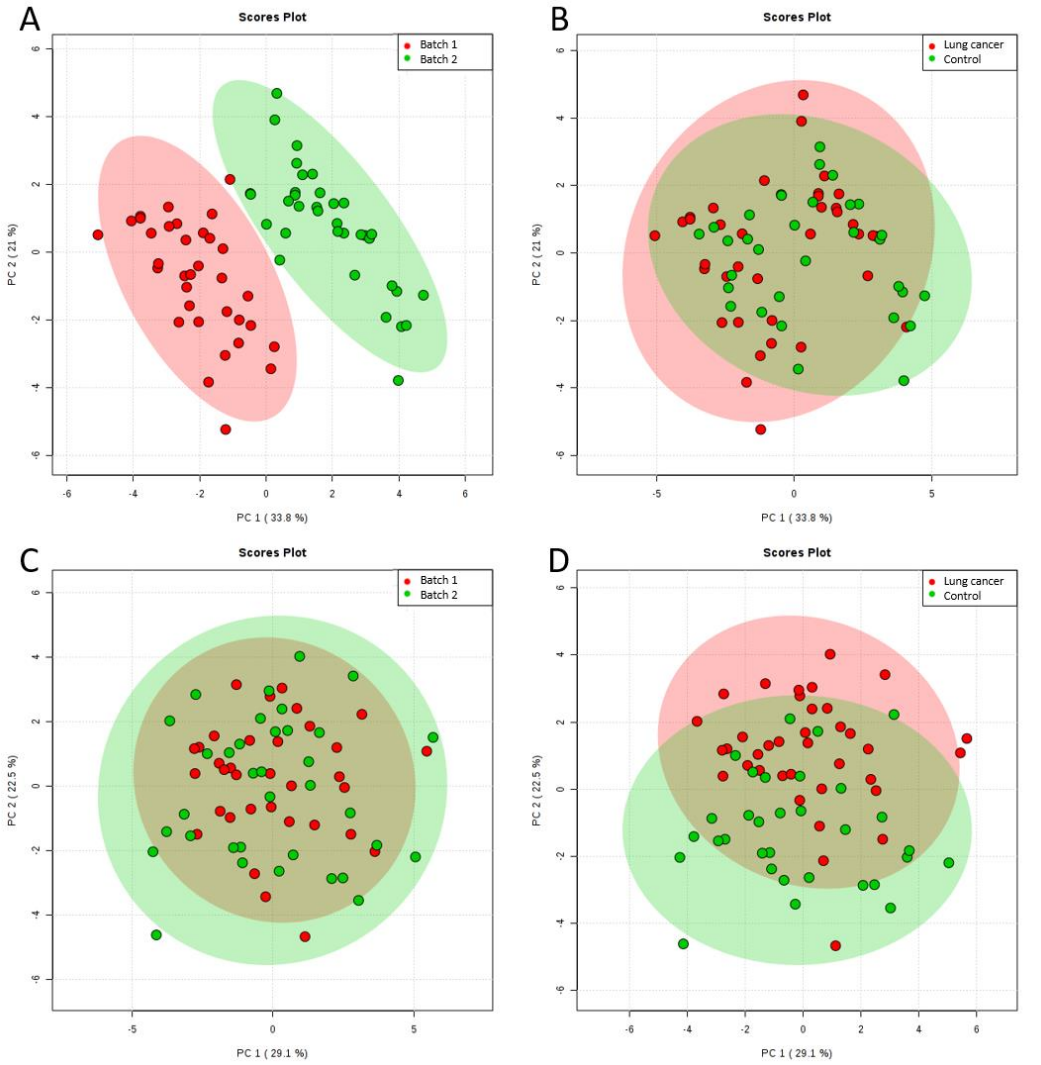

Fig. 1. Two-dimensional PCA score plots after scaling of the complete data set to detect discrimination patterns according to the cohort (**A**) and the diagnostic of lung cancer (**B**). Two-dimensional PCA score plots after separate scaling of the two data sets to detect discrimination patterns according to the cohort (**C**) and the diagnostic of lung cancer (**D**).

3.2. Selection of metabolites with high predictive capability

Random Forest Analysis was used to rank tentative identified metabolites according to their importance, evaluated by measuring the increase of the unbiased estimation of the classification error (Xia *et al.*, 2009). Since metabolites are ranked by their statistical significance for discrimination of individuals between the two groups under

study, this classification can provide evidences on the tentative metabolites characterized by a high prediction capability and, therefore, the most probable to be combined in a panel of markers to discriminate the two classes.

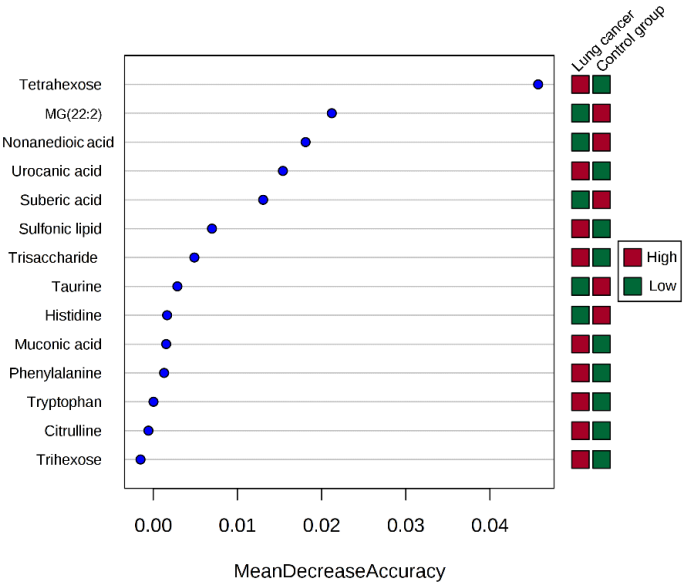

Fig. 2. Random Forest Analysis of significant metabolites found in human sweat to discriminate lung cancer patients and risk factor individuals.

Fig. 2 shows the first 14 metabolites ranked by their contributions to classification accuracy. As can be seen, tetrahexose, MG(22:2), nonanedioic acid, urocanic acid and suberic acid presented the highest probability to appear in a panel to discriminate lung cancer patients and control individuals, while the only two compounds excluded from the classification were two dipeptides, GluVal and GluLeu that reported, together with the trihexose and citrulline, mean accuracy values below zero. Taking into account the significance of the metabolites, a Kruskal-Wallis test was applied to the fourteen compounds detected in each batch and in both batches together. As can be seen in Table 2, among the seven significant metabolites found in the first batch (Calderón-Santiago *et al.*, 2015), only one is significant in the second batch, but when the two batches are taken together, only one metabolite is excluded from the list of significant metabolites (the trihexose). Therefore, the inclusion of the second batch did not alter the changes in metabolite composition between lung cancer and risk factor individuals. Supplementary Fig. 2 shows the box-and-whisker plots for the 12 metabolites after exclusion of the two

dipeptides with higher classification capability according to Random Forest Analysis. Half of the compounds presented higher quantitative response in lung cancer patients than in control individuals. This is the case with tetrahexose, urocanic acid, sulfonic lipid, trisaccharide phosphate, muconic acid, phenylalanine, tryptophan, citrulline and trihexose. The rest of compounds presented higher relative concentration in control individuals than in lung cancer patients.

Table 2. Kruskal-Wallis statistical analysis to evaluate the significance of the fourteen metabolites selected after the Random Forest test to discriminate between lung cancer patients and risk factor individuals.

Marker	***p*-Value**		
	1st Batch	**2nd Batch**	**1st + 2nd Batches**
Urocanic acid	0.0018**	0.3436	0.0023**
Tetrahexose	0.0020**	0.0597	0.0003**
Trisaccharide phosphate	0.0033**	0.5060	0.0080**
Nonanedioic acid	0.0111*	0.0292*	0.0005**
MG(22:2)	0.0150*	0.4410	0.0152*
Suberic acid	0.0200*	0.0962	0.0025**
Trihexose	0.0343*	0.4410	0.2376
Histidine	0.1613	0.3259	0.0531
Phenylalanine	0.1715	0.0074**	0.3290
Taurine	0.1823	0.7267	0.4839
Citrulline	0.2858	0.5288	0.1770
Sulfonic lipid	0.3011	0.0548	0.0249*
Tryptophan	0.6353	0.8614	0.8436
Muconic acid	0.7811	0.1584	0.2523

* *p*-Value between 0.01 and 0.05; ** *p*-Value < 0.01.

3.3. Development of panels with prediction capability for lung cancer

The development of panels for discrimination between lung cancer patients and risk factor individuals was addressed by using PanelomiX as computational toolbox. This tool iteratively combines significant variables and thresholds to find panels of potential biomarkers with good prediction/discrimination parameters in terms of sensitivity and

specificity. As mentioned above, the most important aspect in a prediction model for lung cancer is to reduce the probability of false negatives, while the probability of false positives is not so critical as the disease could be confirmed by other tests. Nevertheless, the development of discrimination models targeted at reducing false positives would also be useful as screening strategy to minimize the number of patients subjected to invasive confirmatory tests. In this research, the configuration of panels was supported on two statistical criteria: at least 95% sensitivity or specificity, which resulted in two panels of biomarkers. The panel with at least 95% specificity should allow decreasing considerably false negative rate. On the other hand, a biomarker panel with at least 95% sensitivity should allow a significant decrease of false positive rate. PanelomiX was applied to the two whole cohorts together (which were previously auto scaled separately, then joined and normalized) to define panels of five biomarkers with the best classification parameters. Metabolites listed in Supplementary Table 1, from both positive and negative ionization modes, were considered as the set of variables, excluding GluVal, GluLeu, trihexose and citrulline, the four compounds with less probability of taking part in a prediction panel according to Random Forest Analysis. Cross-validation was included in the development of panels for internal validation, which reported information on the model stability. The classification capability of the panels was assessed by threshold sensitivity and specificity and the value of the partial area under the curve (pAUC), calculated taking the range of specificity or sensitivity from 95 to 100%. Furthermore, a cross-validation including the scaling procedure was also applied to the optimum panels to assure the correctness of data pretreatment. The best panel providing specificity above 95% was obtained by combination of five metabolites: the monoglyceride MG(22:2), suberic, muconic and urocanic acids, and a tetrahexose. The parameters that characterized this panel were 96.9% specificity, 83.8% sensitivity and the pAUC was 1.6%. This panel would allow diagnosing individuals with lung cancer with a low false positive rate. Fig. 3A shows the receiver operating characteristic (ROC) curve obtained from this panel that included four metabolites present in the top-5 compounds ranked by Random Forest Analysis and that for nonanedioic acid, which was the marker with the highest predictive capability in terms of specificity. The parameters provided by nonanedioic acid were 93.8% of specificity, 45.9% of sensitivity and a pAUC of 1.7%. The specificity of this marker was similar to that provided by the panel, but the sensitivity and pAUC are improved when considering a group of compounds.

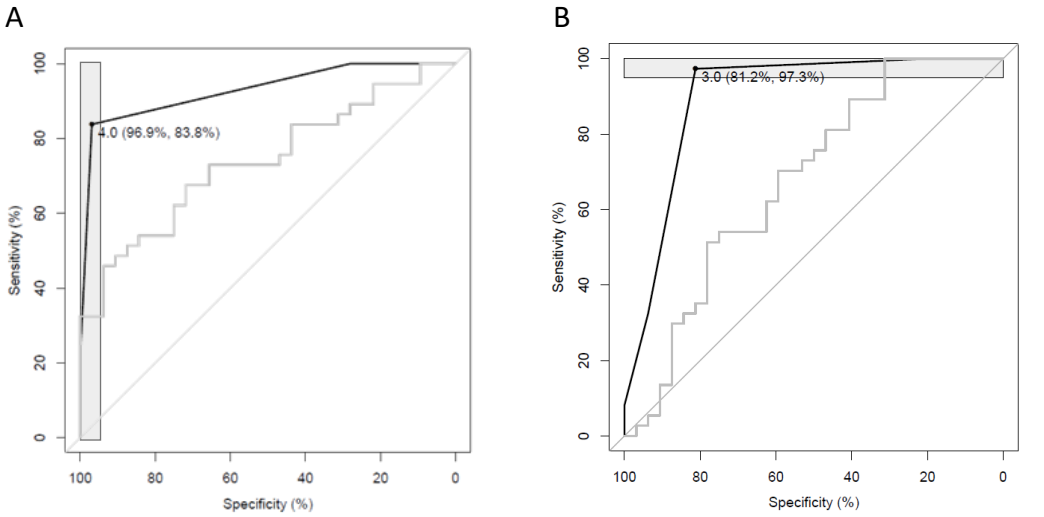

Fig. 3. ROC curves obtained from the panel with at least 95% specificity (black) and that of nonanedioic acid (grey) as the best individual marker in terms of specificity (**A**) and ROC curves from the panel with at least 95% sensitivity (black) and that of MG(22:2) (grey) as the best predictive marker in terms of sensitivity (**B**).

Curiously, although nonanedioic acid showed the third best accuracy response in the Random Forest ranking, and was the metabolite providing the best specificity, it was not included in the panel. This panel reported a positive response when any 4 of the 5 markers (suberic acid, muconic acid, tetrahexose, urocanic acid and MG(22:2)) fulfilled the conditions imposed in the panel. As an example, the quantitative response of suberic acid for a positive response (lung cancer detection) should be below 34.7% of the total variability scale defined by the highest and the lowest quantitative responses for this metabolite in the two cohorts. By analogy, the MG(22:2) must be below 78.8% of the total variability scale, while the levels of urocanic acid, muconic acid and tetrahexose should be above 53.0, 38.2 and 19.3%, respectively (Table 3).

Fig. 4A shows the box-and-whisker plots of the five metabolites that constitute the panel with at least 95% specificity with a distribution of quantitative responses detected for all individuals. The dashed lines pinpoint the quantitative response imposed by the panel for a positive response. As can be seen, the threshold stablished for MG(22:2) allowed classifying correctly 100% of controls.

Table 3. Panels of biomarkers with at least 95% of either specificity or sensitivity.

Panel 1 (>95% specificity)			**Panel 2 (>95% sensitivity)**	
Urocanic acid	> 53.00%		Urocanic acid	> 50.31%
Muconic acid	> 38.23%		Muconic acid	> 43.59%
Suberic acid	< 34.71%		Nonanedioic acid	< 45.53%
MG (22:2)	< 78.79%		MG (22:2)	< 78.79%
Tetrahexose	> 19.31%		Tetrahexose	> 91.55%

Both panels provide a positive response when 3 of the 5 biomarkers of each panel give a quantitative response below or above the corresponding threshold.

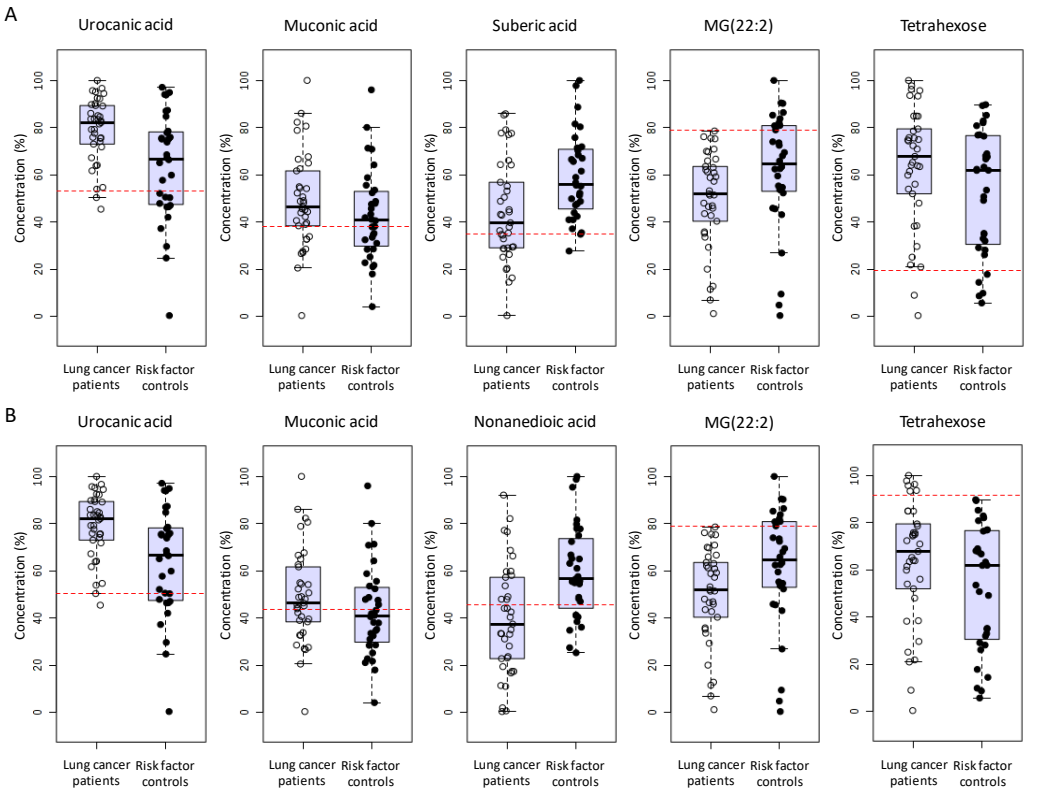

Fig. 4. The box-and-whisker plots of the five metabolites that constitute the panel with at least 95% specificity (**A**), and with at least 95% specificity (**B**) with distribution of quantitative responses for all individuals. The *y* axis represents the normalized concentration of the metabolites expressed at percentage according to the samples with the highest and the lowest quantitative response. The dashed lines pinpoint the quantitative response of each marker to provide a positive response.

Similar specificity was obtained for urocanic acid and tetrahexose, 94.6%, followed by muconic acid that classified correctly 75.7% of controls. On the other hand, suberic acid gave the best performance for lung cancer patients, classifying correctly 96.8% of them. It is worth mentioning that this panel included three of the compounds contained in that obtained by Calderon-Santiago *et al.* using only the first batch. The prediction parameters, sensitivity and specificity, were clearly higher than those reported in the first study. Thus, the first panel provided 80 and 79% for specificity and sensitivity, respectively; while the new panel proposed here increased especially the specificity that reached 96.9%. The inclusion of the second batch improved significantly the capability of the panel to identify individuals without the disease and, therefore, they would be excluded from the application of a confirmatory test.

On the other hand, the best panel providing sensitivity above 95% was obtained only by replacement of one of the metabolites included in the previous panel, suberic acid by nonanedioic acid. It is worth emphasizing that suberic acid was included in the panel of three markers obtained by Calderon-Santiago *et al.* maximizing specificity, while nonanedioic was obtained in that targeted at maximizing sensitivity. Fig. 3B shows the ROC curve obtained for this second panel together with that obtained for MG(22:2), which provided the highest individual prediction capability in terms of sensitivity. The parameters obtained for this panel were 81.2% specificity, 97.3% sensitivity, and a pAUC of 1.6%. In this case also four of the markers included in the panel were part of the top-five metabolites in the Random Forest ranking. MG(22:2) presented 31.2% specificity, 100.0% sensitivity, and a pAUC of 1.6%, being the second best accuracy value in the Random Forest ranking. This panel contained also three of the metabolites included in the panel obtained in the study published in 2015. With the inclusion of the second batch, the new proposed panel allowed increasing the sensitivity up to 97.3%, which means that the number of cases affected by the pathology would be identified with a high detection rate.

This panel was positive when any 3 of the 5 biomarkers (urocanic, nonanedioic and muconic acids, tetrahexose and MG(22:2)) gave a positive response. For this purpose, the quantitative response of MG(22:2) and nonanedioic acid must be lower than 78.8 and 45.5% of the total variability scale defined by the highest and the lowest quantitative responses observed for this metabolite in the two cohorts, respectively. Similarly, the quantitative responses of muconic and urocanic acids, and tetrahexose must be higher than 43.6, 50.3, 91.5%, respectively (Table 3). Fig. 4B shows the box-and-whisker plots of the

five metabolites that constitute the panel with at least 95% sensitivity and the cut-off values imposed by the panel. As can be seen, tetrahexose classified correctly 100% of lung cancer patients, followed by nonanedioic acid with 75% of good performance for lung cancer patients. On the other hand, MG(22:2) classified properly 100% of risk factor individuals, followed by urocanic acid with 97.3% specificity and nonanedioic and muconic acids, both classifying correctly around 62% of controls.

As stated above, a cross-validation process including the scaling procedure was executed for both optimized panels as described above. For the panel optimizing specificity (composed by tetrahexose, MG(22:2) and suberic, muconic and urocanic acids), the new panels generated with only 90% of the samples gave from 81.8 to 96.9% sensitivity and from 82.8 to 96.9% specificity. Furthermore, the 10% of the samples excluded gave 62.3% sensitivity and 70.8% specificity. Similarly, for the panel optimizing sensitivity (including MG(22:2), tetrahexose and muconic, urocanic and nonanedioic acids), a sensitivity from 96.9 to 100% and a specificity from 72.4 to 79.3% were obtained for the 10 panels generated with only 90% of the samples. Then, a sensitivity and specificity of 86 and 51% was obtained when testing the panels in the samples excluded in the panel generation process.

3.4. Biological interpretation of significant metabolites included in the panels

Regarding the markers included in the panels, it is worth emphasizing the presence of short chain dicarboxylic acids such as muconic, suberic or nonanedioic acids. The last is a peroxidative lipid breakdown product, the levels of which appeared in a recent work to be lowered in blood samples from lung cancer patients as compared to smokers (Miyamoto *et al.*, 2015). In sweat, nonanedioic acid was found in lung cancer patients at lower concentration than in risk factor individuals. Nonanedioic acid is supposed to be linked to signaling functions or a reflection of a lowered use of oxidative pathways of lipid metabolism in tumors (Miyamoto *et al.*, 2015). The levels of suberic acid and nonanedioic acid in urine have been previously proposed as potential markers to discriminate patients with cervical cancer (malignant tumor) and patients with uterine myoma (benign tumor) (Kim *et al.*, 1998). Suberic acid was found at lower concentration in cervical cancer patients whereas nonanedioic acid was found at higher concentration in cervical cancer patients (Kim *et al.*, 1998). On the other hand, the level of muconic acid has been previously related

to the exposure to benzene (Zhang *et al.*, 2011) —for example, in smokers (Wiwanitkit *et al.*, 2005)— a factor already linked to cancer development (Snyder, 2012).

Other metabolite included in the panels was urocanic acid, a degradation product from histidine. The levels of several amino acids in plasma, including histidine, together with the levels of other compounds such as lipoproteins, glucose, lactate, pyruvate, citrate, formate, acetate, and methanol have also been previously proposed for discrimination lung cancer patients and healthy subjects (Rocha *et al.*, 2011). Additionally, some amino acids in urine have been identified as potential biomarkers for lung cancer, which revealed active amino acid and nucleoside metabolism as well as protein degradation in lung cancer patients (An *et al.*, 2010). Urocanic acid, the product of histidine degradation, is a UV filter found in human skin that protects this from UV damage, but has also been linked to the onset of skin cancer and to photoimmunosuppresion (Tuna *et al.*, 2014). Some studies suggested that urocanic acid can modulate the production of immunosuppressive molecules from keratinocytes, nerves and mast cells (Hart *et al.*, 2002; Kaneko *et al.*, 2008; Khalil *et al.*, 2001).

Urocanic acid had been previously used to build a panel, together with other nine compounds (proline betaine, pyridylacetic acid, 1-methylguanine, uric acid, methyl-xanthine, tryptophan, theophylline, and carnitine C8:1) for detection of uterine cervix cancer using urine as sample. The panel provided an AUC of 0.997, with a sensitivity of 92.9% and a specificity of 95.6% (Chen *et al.*, 2013).

Other component of the panels was the monoglyceride (MG) of an omega-6 fatty acid —docosadienoic acid—, which is involved in a critical biochemical pathway as is the metabolism of essential fatty acids (Simonsen *et al.*, 1998; Simopoulos, 2002; Simopoulos, 2008). The levels of some MGs have been demonstrated to be altered in tumor tissues (Yang *et al.*, 2015). Finally, another metabolite involved in the panels was a carbohydrate, particularly a tetrahexose. The presence of these compounds is related to amylases, enzymes present in most human biofluids, including sweat, and whose composition and action has shown to be altered in tumor tissues (Takeuchi *et al.*, 1981).

As stated above, this research employed a first cohort that was already used to find a tentative panel of markers for lung cancer diagnosis. When this panel was compared with panels obtained using two cohorts in the present research, the panel that maximizes specificity and sensitivity differs in two metabolites with the panel obtained with only one

batch. The two panels obtained in the present research contains 4 compounds in common: MG(22:2), tetrahexose and urocanic and muconic acids, from which the two first compounds were present in the previous research. It is worth emphasizing that these two compounds are the two best compounds according to their accuracy in the Random Forest test. Nonanedioic and suberic acids, compounds exclusive for the panels maximizing sensitivity and specificity, respectively, were also present in the panel obtained only with the first batch. The novel presence of urocanic and muconic acids, as well as the exclusion of trihexose, can be attributed to the inclusion of the new cohort, which not only included samples analyzed at different times, but also implies slightly different percentage of histological types and stages.

These preliminary results emphasize the necessity of a large-scale study to validate the proposed panels and obtain great robustness. The use of these panels would tentatively reduce the proportion of individuals to be subjected to confirmatory tests and to detect lung cancer in less advanced stages. However, the efficiency of the panels should be demonstrated with the inclusion of new batches.

4. Conclusions

A strategy to remove instrumental variability between batches has been proposed and satisfactorily applied to the two cohorts employed in the present research. The use of batches of samples collected and analyzed with a shift time of two years and by different analysts would allow to obtain a prediction model more robust than that obtained using the first batch. Two panels of metabolites including amino acids, short chain dicarboxylic acids, sugars and some lipids have been built to discriminate patients with lung cancer from a control group with risk factor. The best panel providing specificity above 95% was formed by five metabolites, the monoglyceride MG(22:2), muconic, urocanic and suberic acids, and a tetrahexose. This panel provided 96.9% specificity and 83.8% sensitivity. On the other hand, the best panel providing sensitivity above 95% was formed by four of the metabolites included in the previous panel, so only suberic acid was replaced by nonanedioic acid. This panel provided 81.2% specificity and 97.3% sensitivity.

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Compliance with ethical standards

Ethical approval

The study was approved by the ethics committee of the medical faculty at the University of Córdoba and was in accordance with the 1964 Helsinki declaration, and study participants provided their written informed consent.

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Supplementary material

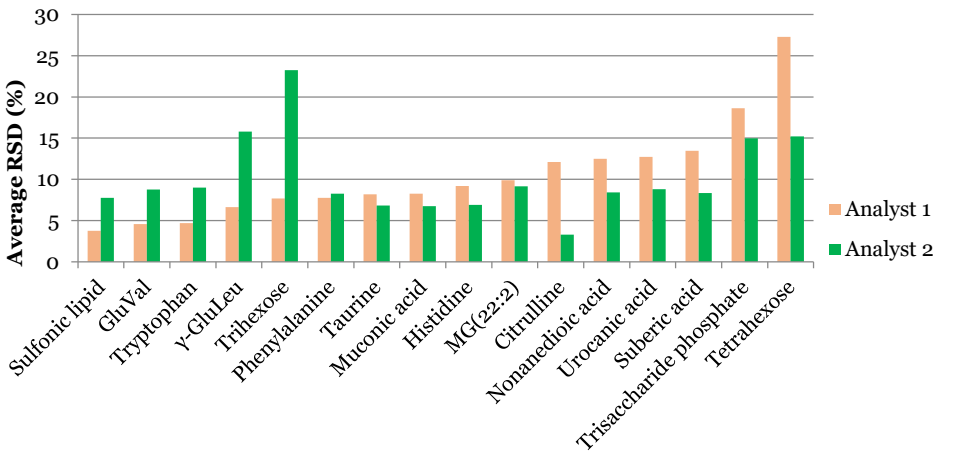
Supplementary Table 1. Metabolites found in sweat at significantly different concentrations between the groups under study including the retention time and *m/z* values for precursor and product ions as well as the error in precursor ion detection expressed as ppm.

Negative ionization mode

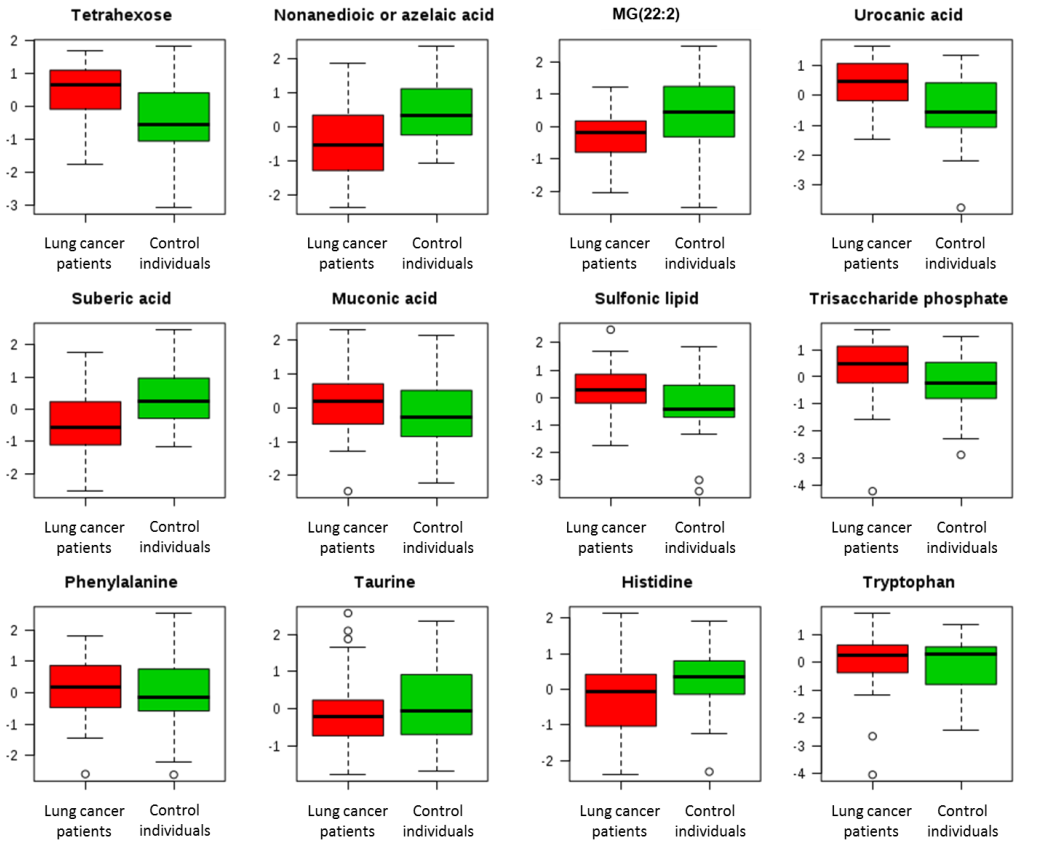
	Compound name	HMDB ID	RT (min)	Precursor ion (*m/z*)	Δppm	Product ions (*m/z*)
1	Citrulline	HMDB00904	0.8	174.0883	0.6	131.0818 44.9974 72.0078
2	Trisaccharide phosphate		1.0	601.1371	1.7	547.0835 96.9696 161.0440 259.0221
3	Phenylalanine	HMDB00159	4.1	164.0718	0	147.0445 103.0549 91.0549 72.0094
4	Nonanedioic acid	HMDB00784	7.1	187.0969	3.7	125.0962 187.0954 97.0645 57.0335
5	Sulfonic lipid		8.4	577.1470	1.6	170.0046 391.1109 497.1911
6	Trihexose		0.8	549.1672	0	503.1598 341.1085 179.0553 89.0241
7	Histidine	HMDB00177	0.8	154.0619	2	137.0358 93.0457 80.0385 67.0307
8	Taurine	HMDB00251	0.8	124.0073	0.8	79.9567
9	Urocanic acid	HMDB00301	1.4	137.0357	0	93.0445 66.0349 65.0206 50.0050
10	Muconic acid	HMDB06331	7.7	123.0089	5	53.0033 95.0139 68.9982
11	Suberic acid	HMDB00893	6.6	173.0824	2.9	111.0809 83.0503 57.0349
12	MG(22:2)	HMDB11581	8.9	431.3127	3.6	89.0247

Positive ionization mode

	Compound name	HMDB ID	RT (min)	Precursor ion (*m/z*)	Δppm	Product ions (*m/z*)
1	Tryptophan	HMDB00929	4.7	205.0971	0.5	188.0699 146.0593 118.0647
2	Tetrahexose		0.8	689.2113	0.3	145.0488 127.0385 85.0283 487.1640
3	GluVal	HMDB59717	4.6	229.118	3.9	183.1104 118.0852 72.0809 102.0550
4	γ-GluLeu	HMDB29153	4.6	261.1439	2	244.1169 132.1014 86.0968 84.0443



Supplementary Fig. 1. Mean relative standard deviation (RSD) obtained for each metabolite considering the replicates analyzed per sample by analyst 1 and 2.



Supplementary Fig. 2. The box-and-whisker plots of the metabolites with accuracy above 0 for the comparison between lung cancer patients and risk factor individuals. The bottom and top of the boxes represent the 25th and 75th percentile, respectively. The top and bottom bars represent the entire stretch of the data points for the subjects, except the extreme points, which are indicated with circles (o). The hyphen indicates the median value. The y axis represents the normalized relative concentration of the metabolites.

Discusión de los resultados

Discussion of the results

The present rules in the University of Córdoba concerning the model of Thesis-Book in the format in which the articles as such (either published or close to be published) are included in the book, make mandatory to include a joint discussion of the obtained results.

The research in this Thesis-Book, based on sample preparation and on platforms development for both plant and animal metabolomics analysis, including US as auxiliary energy to favor analytical steps, has been divided into four sections as a function of the pursued objectives. Thus, Section A, constituted by Chapters I-to-III, contains critical reviews and discussion on the state-of-art of the US as auxiliary energy on sample preparation steps, with special emphasis on: (i) US devices, (ii) USAE of food/plant components, (iii) US-assisted steps in metabolomics, and (iv) behavior of enzymes when subjected to US application. Also, a large discussion on the effect of US on enzyme-catalyzed reactions when metabolites act as substrates is a part of this section.

The experimental research that constitutes Sections B deals with the appropriate sample preparation and design of the analytical platform to favor by US the enzymatic hydrolysis of oleuropein in olive (*Olea europaea* L.) leaf extracts to oleuropein aglycon, a compound that may have a pharmacological use (Chapter IV); and to elucidate the metabolome by untargeted analysis of extracts of two different plants with clinical applications: edible mushroom *Agaricus bisporus (A. bisporus)* aqueous enzymatic extracts with antiviral activity against HCV (Chapter V), and *Cannabis sativa* L. extracts (Chapter VI).

The experimental research that constitutes Sections C and D deals with the appropriate sample preparation and design of the analytical platforms to elucidate the metabolome of human sweat by GC–MS (Chapter X), after comparing different sampling strategies (Chapters VIII and IX). It also deals with the appropriate sample preparation and design of the analytical platform to obtain robust panels of metabolites for screening of lung cancer using human sweat as sample (Chapter XI) and to quantify in this biofluid amino acids, biomarkers of lung cancer (Chapter VII), both by LC–MS/MS.

Section A. Bibliographical studies

To start with, and during the development of this PhD Thesis, bibliographical studies were a continuous and key task to focus the research on the current needs in the given area. Thus, the growing application of USAE to plant/food components, the use of US to improve sample preparation steps in metabolomics and the effect of this auxiliary energy on enzymes and their biocatalytic action have been critically revised and discussed.

Chapter I of this Thesis-Book is devoted to critical review of the increased application of USAE to foodstuffs. A general overview on the US basics and its features that assist in extraction, the extractants and US devices used are covered. As described in this chapter, the low-frequency region of US is endowed with appropriate energy to produce cavitation, highly dependent on frequency and of great interest for most of the sample preparation steps. US favors mass transfer between two phases in the same or different physical state, accelerating and/or improving physical phenomena and chemical reactions [1]. In dealing with plant tissues, US application implicates cell membrane destruction, causing morphological changes of the plant structure, and possibly with drastic change of its properties. Regarding extractants, their features influence considerably USAE efficiency, which is also highly dependent on the nature of both the matrix and analyte. Two main types of US equipment, ultrasonic cleaning baths and probes, have been considered in this chapter. The disadvantages of baths as compared with US probes (*e.g.*, no uniform ultrasonication with reproducibility problems or declination of the transferred power into the tank over time), the versatility of power and duty cycle programming as required, which with probes are endowed, make these better suited to facilitate analytical steps in laboratories than US cleaning baths.

Characteristic examples of USAE methods used for food components extraction (*e.g.*, antioxidants, volatiles, multielements, undesirable compounds) are critically debated in this chapter. As commented in it, US accelerates extraction, improves extraction yield, and facilitates automation; favorable aspects that have been exploited to extract families of compounds with specified characteristics from a raw material, to be used as nutraceuticals. One common aspect of most of the USAE methods in the literature is monitoring of the extraction process exclusively by global parameters of the extract such as antioxidant activity, total phenols content, etc. [2]; while degradation of the extract components under

US application is only detected by a decrease of the measured absorbance by a photometer. What is emphasized in Chapter I on this subject is the necessity of identification of the extract components, particularly when the extracts are devoted to the food industry, where the nature of the degradation products should be well known. Other aspect critically discussed refers to volatiles extraction, which can be favored by US: it is known that this energy helps to remove a gas from a liquid, as used to obtain mobile chromatographic phases free from air. Nevertheless, when the gas phase is the target, especial care must be adopted, which was not the case in the extraction of volatiles from saffron, developed in an open atmosphere without protection for losses of both the target compounds, which are transfer to a liquid, and the volatile medium (an ethanol–ethylacetate mixture) [3]. Metal ions have been extracted from food by US assistance prior to determination by the proper atomic detector [4,5]. Also fast and efficient extractions of undesirable compounds from foods have been helped by US [4].

Methods based on the use of both ionic liquids (ILs) and USAE have been applied by taken advantage of a typical US property of favoring emulsification simultaneously with extraction —both involving not only solids but also liquid samples—; and also development of USAE with either simultaneous derivatization or enzymatic catalysis are discussed in Chapter I. An example on the use of ILs is the USAE of four biphenyl cyclooctene lignans from the fruit of *Schisandra chinensis Baill*. After optimization of US power, time for US treatment, and solid–liquid ratio by response surface methodology, the extracts were compared with those obtained by conventional extraction, resulting in an efficiency increase of about 350%, and a shortening of the process time from 6 h to 30 min [6]. Lignans concentration was monitored by an HPLC–UV-detector at 220 nm.

US-assisted emulsification–extraction (USAEE) has been used with two main types of samples: (i) With solid samples and using two immiscible polar/no polar extractants for simultaneous removal of both polar and no polar sample components, as the formation of an emulsion allows a rapid mass transfer of the compounds extractable to one or the other liquid phase under US application. (ii) With liquid samples for liquid–liquid microextraction, for which a large volume of usually aqueous sample is put into contact with a very small volume of organic extractant that is emulsified with the sample under US application.

A very drastic reduction in time required for a reaction assisted by US energy is the result of a study on oil stability reported by the UCO research team that deserves be

discussed. The use of a probe and the appropriate optimization of the US variables allowed Rancimat times of 129 h to be shortened to 50 min; therefore, the overall time required for the determination of oil stability is shorter than 1 h [7]. On the other hand, the debatable effect of US on the enzymatic activity requires in depth studies involving the US frequency and the biocatalyst structure, as shown in the review by Delgado-Povedano and Luque de Castro [8] that promoted the study on the enzymatic hydrolysis of oleuropein to its aglycon form assisted by US, which is the matter of Chapter IV of this Thesis-Book.

The advantages and disadvantages of USAE methods have also been compared in this chapter with those of other conventional or unconventional extraction methods as the case of Soxhlet extraction (SE) and USAE to remove vanillin from cured vanilla pods that allowed to short the extraction time from 8 to 1 h [9]. USAE also proved to be better than percolation and Soxhlet methods in terms of efficiency to extract 26 characteristic components in ginkgo seeds [10]. Also, the combined effect of sequential application of US and MW (US–MAE) on the yield and degree of esterification of pectin from pomelo peel using citric acid [11] has been checked and the order of yield efficiency was US–MAE > MW–USAE > MAE > USAE. The pectin galacturonic acid obtained by combined extraction techniques exhibited a pseudoplastic behavior, and the yield was always higher than that provided by a single technique.

The exploitation of USAE at industrial and pilot scale is also considered, but pointing out that the use of US in the food industry is not exclusive of extraction, as filtration, defoaming, depolymerization, degassing/deaeration, cooking, cutting, demolding and extrusion, freezing and crystallization, drying, defrosting/thawing, brining, pickling and marinating, meat tenderization, separation (emulsion breaking), and emulsification/homogenization have also been favored by this type of energy. When considering US for extraction purposes, an exhaustive development at the laboratory scale is mandatory, particularly for identification of the extract components, with an especial emphasis on the potential degradation products caused or favored by US energy. Also residues or waste valorization has been a remarkable application of USAE over the last decade [12,13].

The in-depth study of the literature on USAE allowed concluding with the following foreseeable and desirable future trends: (i) to develop USAE methods with exhaustive identification of the extract components to detect any potential degradation; (ii) to pay attention to US frequency, because to test different frequencies of US devices can be very

useful to avoid degradation and/or obtain the pursued results; (iii) to carry out additional research, including upscale studies, to facilitate the adoption of US in the bioactive compounds industries —thus enhancing processing efficiency and capability after in-depth study of extractants, thermolability, and fragility against US action, particularly against free radicals formed in polar media; (iv) to apply multivariate optimization designs to reduce the number of experiments, but also use univariate optimization of some parameters, of paramount importance to assess the effect that changing process variables has on the target components; (v) to get a more in-depth knowledge of USAE at the laboratory scale to potentiate the interest in upscaling methods, thus taking advantage of their positive aspects at industrial scale.

Metabolomics has also taken advantage of US to improve sample preparation (SP) steps, as shows Chapter II. The operations to be developed prior to introduction of the analytical sample either into high-resolution separation equipment or into the detector in metabolomics are very varied, and most of them can be accelerated/improved by US [1].

As shown in the literature on the subject, US has been mainly used in targeted metabolomics analysis (*e.g.*, determination of estrogens in human urine [14]), but US has begun to be used in other strategies, as global metabolomics profiling (*e.g.*, metabolomic profiling of human saliva [15]), which highlight US subexploited utility.

The research on the US–metabolomics binomial reported in this chapter reveals its dissimilar development depending on the area (plant or animal) and the sample preparation step. Plant metabolomics and US assisted leaching (USAL) has received the greatest attention (encompassing subdisciplines such as metallomics, xenometabolomics and, mainly, lipidomics), but also liquid–liquid extraction (LLE) and (bio)chemical reactions in metabolomics have taken advantage of US energy. Among plant metabolites, lipids and other bioactive compounds such as antioxidants have received the highest attention, but also leaching prior to the determination of metals in plants has been performed [16]. Regarding lipids, US has been widely used to improve leaching of a wide number of metabolites as triglycerides and fatty acids, thus constituting a key tool for sample preparation steps in lipidomics [17,18]. Although most samples subjected to USAL have been seeds [19], also flowers and other plant parts have been the target of this step. USAL has also been compared with other conventional techniques as SE [19] or

supercritical fluid extraction, and in all instances USAL was found to shorten the leaching time for the same or similar target metabolites [19,20]. The number of applications of US to favor leaching of metabolites different from lipids in dealing with plants drastically surpass other types of matrices, and, among metabolites, phenols and antioxidants in general have been the most frequent targets. The final use of the leachates establishes both the optimum features of the method and the analyses to be carried out. One of the most used global methods for determination of the total phenolic content is based on the Folin–Ciocalteu reagent, which has been applied to USAL leachates from different plants as apple pomace, arecanut, black chokeberry, olive leaves or grapes. In the last case, other general methods such as those for total amount of anthocyanins and total amount of tannic components have also been applied to the leachate. In all overall studies, higher values of the given parameter were obtained when the leaching step was assisted by US as compared with traditional leaching methods. This behavior was attributed to a higher efficiency of the USAL step without checking possible degradation in the presence of power US that could yield products with higher values of the parameter under study. Research on this subject should involve, (i) appropriate analytical instrumentation to identify the compounds in the leachate thus detecting potential degradation; (ii) use of higher US frequencies to avoid degradation, if undesirable.

When the aim of the research was targeted metabolomics [21,22], drastic reduction of the leaching time was highlighted in all instances, thus opening a door to potential implementation at an industrial scale.

When a single leached metabolite is the aim of the research, a first study should involve the behavior of the target compound when subjected to US action under the possible working conditions. This was the case with the use of several US frequencies (20, 60 and 100 kHz) and four US powers (3.2, 8, 30, 56 W) to study their potential degradation effects on a hesperidin standard prior to be leached from *Citrus reticulata* Penggan peel [23].

Ionic liquids as leachants for USAL have been scarcely reported, but they seemed to be very promising for removal of three terpenoids indole alkaloids from *Catharanthus roseus* [24], using (1-allyl-3-methylimidazolium) Br.

Sample preparation in plant xenometabolomics has also taken profit from US, as is the case of USAL of herbicides from resistant and susceptible plants for subsequent study of the metabolic pathways in each case [25,26].

Clinical and animal samples have also benefited from US-assisted sample preparation in metabolomics studies but in a lesser extension, despite that examples as the following show the advantages of application of this type of auxiliary energy, and should promote its use. A US device (40 kHz and 125 W) has been used to accelerate leaching of bufadienolides such as bufalin, cinobufagin and resibufogenin from toad venom samples prior to individual separation–determination by HPLC–DAD. A yield similar to that provided by USAL in 20 min required 6 h by SE, and 18 h by maceration [27].

Despite US application favors mass transfer between immiscible phases drastically shortening the extraction time, and enhancing sensitivity as a result of the favorable effect of sample–extractant volumes ratio, few applications involving US, LLE and metabolomics appear in the literature. Maybe this situation is due to the fact that, despite the use of US accelerates the LLE step, it does not avoid the need for previous steps mandatory to remove proteins and other high-molecular weight components of biofluids [28].

Regarding US and (bio)chemical reactions in clinical metabolomics, US has been used to enhance hydrolysis of metabolites as sialic acids, and enzymatic hydrolysis (using mainly β-glucuronidase), with drastic acceleration of this slow step [15]; nevertheless, few applications of US in this field have been reported [29].

The main effects of US on metabolomics clinical sample preparation have been shortening of the time required for the given step, and/or increase of its efficiency or availability for automation; nevertheless, as in other areas, the attention paid to potential degradation caused and other effects caused by US has been scant or nil. Therefore, the undesirable action of US energy in dealing with clinical samples should be widely studied with three main aims: (i) to know the nature of the degradation products, and thus the pathways through which degradation occurs; (ii) to prove the influence of the free radicals formed by influence of cavitation; (iii) to study the effect of US frequency on the degradation processes by the decrease of cavitation taking place by gradual change of this variable from power to high frequencies.

In short, while US has been widely used to improve steps of a wide number of plant origin samples, this energy has been scarcely applied to enhance steps of target metabolites from animal or human samples, possibly owing to the fact that clinical personnel are unfamiliar with US. Also, US has been used more in clinical targeted than in untargeted

analysis. USAL at different US frequencies could provide greater selectivity for metabolites families; thus being in the clinical field of similar interest as in the plant field.

The last chapter of this section (Chapter III) was a critical review on the effect of US on enzymes and their biocatalytic action. This review is mainly aimed at showing: (i) the different areas in which the US–enzyme binomial has been applied; (ii) the lack of enough information provided on the US–enzyme-working conditions under which each piece of research has been developed; and (iii) the necessity for providing complete information on the data and metadata to give enough light on each piece of research and thus, on the potential comparison of results from different studies. Advances in this field are closely related to the possibility of comparing the results obtained by different authors under different/similar but well-known working conditions.

The controversial results found in it can be attributed mainly to the different US frequencies, intensities and conditions applied (no specified in the publications), and/or to the different types of enzymes on which US energy is applied and even to the different working conditions. In fact, the no reproducible performance of US cleaning baths and the decline of power with the working time are not taken into account by the authors, who do not consider these aspects affect the results [30]. It is worth emphasizing the growing number of analytical chemists who use US probes or reactors designed for specific uses.

Most of the studies involving enzyme–US can be divided into six groups, in a growing order attending to the information provided on the characteristics of the US device used: (i) without information; (ii) with information on the model of the device; (iii) with information only about US power and/or intensity applied; (iv) with information about frequency; (v) with enough information about the characteristics of commercial conventional US devices; and (vi) with information about no conventional US devices. The needed information is frequency and either, power or intensity for probes or baths. Additional information on the surface area of the US transducer (usually fitted at the bottom) is sometimes given in dealing with US baths. In some research using both a bath and probe, information on frequency and power of the bath is given, but only the model in the case of the probe [31].

Regarding the behavior of enzymes when subjected to US application, it is a proved fact that enzyme performance is greatly affected by the nature of the reaction medium [32].

This effect is enhanced in the presence of US, but it has not been studied and/or discussed in detail by researchers. Special mention deserves the use of ionic liquids (ILs) in the reaction medium because their characteristics dramatically influence the US–enzyme behavior most times.

Most enzymes involved in the biocatalyst–US binomial have been hydrolytic enzymes (particularly lipases, amylases or proteases), either immobilized or in solution. It is worth emphasizing that comparison of research developed with different US equipment acting on enzymes has not been conclusive so far (especially when US is applied on different enzyme–substrate systems and in different media); nevertheless, a common behavior is inactivation of the enzyme in some proportion; an effect that increases by increasing power or intensity, but can be differently affected by US frequency.

The type of reaction more widely assisted by the US–enzyme binomial in dealing with metabolites is hydrolysis. As compared with conventional methods for oil hydrolysis, enzymatic hydrolysis can be performed under relatively milder and simpler process conditions, and enhanced by US energy [33].

From the point of view of the reaction components, division as a function of the substrates involved instead of biocatalysts is more appropriate for a better interpretation of the influence of these components on the effects caused by the US–enzyme binomial. Most of the slow biocatalyzed reactions that have been intended to be accelerated by US energy during more than 20 years have involved lipids, the products from which are applied on different fields (*e.g.*, foods, cosmetics, pharmaceuticals, biofuel). The variety of the substrates and products in each field is so wide that general guides on the behavior of them when subjected to US energy have not been established yet. Esterifications constitute one of the most representative groups of enzymatic-assisted reactions the slowness of which has promoted the study of US application for acceleration [34]. The effect seems to be dependent on US amplitude, the presence of water in the reaction medium and length of the chain of the alcohol involved. Studies on acylations and transacylations attributed the accelerating effect of US to the increase in temperature this energy causes, which also degrades the biocatalyst over a certain value. The increased pressure and active surface of the enzyme were also considered influential variables [35]. After more than 20 years, the results obtained cannot allow general application of given working conditions. Therefore, each piece of research establishes the conditions for working on given systems, for given

ranges of the influential variables and for given optimization designs, either uni- or multivariate approaches.

More recent is the contribution of US-assistance to biocatalyzed biodiesel (BD) production that foreseeably will be place to abundant and fruitful research. A higher BD yield (96%) was obtained after US application to the immobilized Novozyme 435 (using Nagchampa oil and methanol as raw materials in the presence of water) different US frequency, power and duty cycle; therefore, comparison of the results was impossible [36].

Polymers have been subjected to the combined application of US and enzymes mainly to facilitate leaching of metabolites, desirable or undesirable compounds, fractionation of the polymer into smaller polymers or monomers, etc [37,38].

Proteins as substrates of US-assisted enzymatic catalysis was widely covered by Bermejo *et al.* in 2004 in a review on enzymatic digestion and ultrasonication [39].

Furthermore, the degradation, inhibition or activation of the biocatalyst under US application, also the effect of US on enzymes production and the main fields of application of the US–enzyme binomial are covered in the review that constitutes this chapter. An example of the potential of the degradation effects of combined US and enzymes to minimize contamination of aquatic ecosystems by pharmaceuticals consisted of degradation of an antihistaminic drug. Using laccase and US (after optimization of parameters such as enzyme loading, temperature, US power and frequency, duty cycle, and speed of agitation) a maximum degradation of 91% was achieved in 7 h, while with the conventional stirring method the degradation was about 13% in 24 h. The pathway proposed for degradation of the target drug was confirmed by MS [40].

Inhibition or activation of enzyme catalysis by US can be either a pursued effect or an undesirable effect [41]. In short, low-frequency US mainly promotes inhibition of enzymatic activity, probably caused by cavitation and shear forces that should be investigated by proper analytical tools to provide information on the conformational changes produced in the biocatalysts as a function of their structure. Additionally, higher US frequencies should be applied to prove their influence that could be very different.

Moreover, US is a useful tool to assist different steps involved in enzyme production. Thus, enzyme production, extraction, purification, and immobilization can be improved by US application [42].

In dealing with the fields of application of the US-enzyme binomial, food (to improve yields of products of great interest in processed foods), environmental (to accelerate sample preparation methods for subsequent determination of toxicants), clinical (from enzyme production and purification, through synthesis or extraction of compounds of clinical interest, to acceleration of sample preparation in dopping control) and industrial (to assist biofuel production, among others) are the general areas of application.

The nomenclature related to US and the way to express the variables and parameters involved in US energy deserve to be carefully revised. The metadata in this area can be of paramount importance when other scientists try to work on the basis established by previous research as a way to advance in the knowledge. Also, the role of US frequencies on the effect caused by this energy as a function of matrix–analyte–enzyme has been poorly considered. The scant studies on this aspect allow evidencing an enormous field of possibilities, which should be solidly established. Finally, analytical equipment different from photometers and titrimeters is mandatory to know the real effect of US on the given target. Despite techniques such CD, SEM, FT-IR are starting to be used, no enough information can be obtained, most times, from the provided data.

As a consequence of the information discused in Chapter III, the research in Chapter IV was developed to open a new door to the huge and indefinite potential of the US–enzyme binomial. The enzymatic hydrolysis of oleuropein in olive leaf extracts to its aglycon was studied by different hydrolases, which exhibited different behavior and a multivariate design for studying the different values of US variables (*viz.*, amplitude, duty cycle, cycle time). The study of a key US variable (frequency) is a pending goal that requires the design of US devices that only differ in this variable and keep constant the others. Attempts to achieve these designs have been unsuccessful so far.

Section B. Plant metabolomics

To contribute to plant metabolomics by opening new key research lines was the *specific objective* of Section B. The hydrolysis of oleuropein to its aglycon in olive leaf extracts accelerated by different hydrolases and US action constitutes the matter of Chapter IV, which opens the door to the complex enzymes–US world in this section. The characterization of edible mushroom *A. bisporus* aqueous enzymatic extracts with antiviral

activity against HCV, and *Cannabis sativa* L. extracts constitutes the matter of Chapter V and VI, respectively.

Oleuropein and oleuropein aglycon, present in olive tree materials and virgin olive oil (VOO), respectively, could be used as nutraceuticals or as components of new functional foods [43] thanks to their beneficial pharmacological properties [44,45]. The use of VOO as a source of oleuropein and/or oleuropein aglycon for preparation of functional foods is not recommended owing to the high price of VOO and relatively low concentration of these minor components. But olive leaves, which are generated as residue from olive pruning and VOO production (12–30 kg of twigs and leaf residues/tree [46]) are almost an ideal raw material to extract these compounds, and also other highly valuable compounds they contain [47]; all them scantly exploited so far [48].

The content of Chapter IV (on the hydrolysis of oleuropein to its aglycon in olive leaf extracts accelerated by different hydrolases and US energy) constitutes an attempt of shading some light on the very controversial results found in the literature on this subject and discussed in Chapter III [8]. With this purpose, a fast US-assisted enzymatic hydrolysis (USAEH) method to obtain oleuropein aglycon from olive (*Olea europaea* L.) leaf extracts with minimum production of more degraded products was developed in this chapter. A drastic shortening of the time required for complete hydrolysis was achieved as compared with the traditional method based on enzymatic incubation, from 2 h to less than 20 min. The enzyme used in both cases was *β*-glucosidase from *Aspergillus niger (A. niger)* and the optimum US conditions for achieving maximum yield of oleuropein aglycon were 0.5 s/s duty cycle, 50% amplitude and 45 s cycle, optimized by a Box-Behnken design, and 10 US cycles (in 18.75 min) at 20 kHz. The method developed using oleuropein standard also allowed to obtain oleuropein aglycon from commercial and laboratory extracts from olive leaves, which may have a pharmacological use because of its healthy properties. A previous extraction of oleuropein from olive leaf extracts was carried out by agitation with a methanol–water mixture, while hydrolysis was carried out by *β*-glucosidase from *A. niger*, and monitoring of degradation and quantitation was achieved by LC–MS/MS with multiple reaction monitoring. The hydrolysis yield of oleuropein was 90.8% after 10 US cycles, which produced 90% of oleuropein aglycon for commercial olive leaves extracts. The hydrolysis yield of oleuropein reached 95.7% at 10 US cycles, with formation of 94.9% of the aglycon form for laboratory olive leaf extracts.

The fast production of oleuropein aglycon thus achieved open a door to the exploitation of the interesting pharmacological properties of this compound [49,50], and its wide use for cosmetics, functional foods and nutraceuticals [43].

Other good candidates to be included in healthy diets as components of new functional foods or nutraceuticals are edible mushrooms and their extracts or fractions of them. Concerning disease prevention, we recently described the "*in vitro*" inhibition of the protease NS3 of hepatitis-C virus (HCV) by aqueous enzymatic extracts of the common edible mushroom *Agaricus bisporus* (*A. bisporus*) [51]. This fact suggests incorporation of these extracts to the diet as components of new functional foods or nutraceuticals for hepatitis-C prevention in people under risk of HCV infection. The use of these extracts as they are, as a part of new functional foods or as nutraceuticals, requires characterization of *A. bisporus* aqueous enzymatic extracts (AbAEE) as exhaustive as possible, with especial emphasis on checking their safety. As far as we know, this characterization had not been carried out. For this reason, the research in Chapter V was developed. A liquid chromatography–tandem mass spectrometry (LC–QTOF MS/MS) platform was used to obtain, for the first time, the data for tentative identification of 55 out of 123 total metabolites by combination of MS and MS/MS information on aqueous enzymatic extracts of the common edible mushroom *A. bisporus*, which had shown inhibition of the protease NS3 of HCV. The tentative identification of as many AbAEE components as possible was performed after an appropriate sample preparation strategy for fractionation based on sequential LLE of AbAEE with solvents of different polarity. The LC–QTOF MS/MS was used in high-resolution mode to obtain the required analytical information, which provided the necessary knowledge for a safe use of the extracts as they are; a very promising fact for HCV patients.

Among the new identified compounds there are amino acids, sugars, carboxylic, fatty and cinnamic acids, mono- and disaccharides, phospholipids, and purines as the most outstanding, thus demonstrating that fractionation based on sequential LLE followed by LC–QTOF MS/MS is a suitable option to obtain a wide, representative snapshot of AbAEE composition.

Another plant, in this case *Cannabis*, is the most widely cultivated, produced, trafficked and consumed drug worldwide, with approximately 183 million consumers in 2016 [52]. Since 1990 the crop of hemp has been introduced or reintroduced in several countries to obtain fibre and grains [53]. It is being explored increasingly for medicinal applications and therapies, particularly one of its main cannabinoids, CBD [54]. In fact, *Cannabis* is used in the treatment of disorders such as migraine, spastic and pain disorders, and glaucoma as ocular hypotensive. Moreover, cannabinoids have been useful as antiemetic [55]. Countries such as Canada, Germany, Israel, the Netherlands and more than 50% of the United States have fully authorized the medical application of herbal *Cannabis* [56].

Most analytical studies on characterization of *Cannabis* plants were targeted at the detection of two chemical families: cannabinoids and terpenoids. The analysis of both families has been addressed with different analytical platforms [57], mainly by a combination of gas chromatographs or liquid chromatographs to mass spectrometers (GC–MS, LC–MS) [58,59]. Also, GC coupled to a flame ionization detector (GC–FID) has been recommended in cases such as the analysis of *Cannabis* and its products by the United Nations [60]. There are scant studies dealing with compounds in *Cannabis sativa* other than cannabinoids and terpenoids [61,62].

Considering the interest on the study of *Cannabis sativa* for the scientific community in fields such as the pharmacological, biomedical, and agronomical (to name just a few), clearly, more research on *Cannabis* composition is demanded to assess its potential for pharmacological use, but also to implement breeding programs with agronomical purposes. In this line, the research in Chapter VI may constitute a key advance in identification of components of this controversial plant. A total of 169 compounds (cannabinoids, terpenoids, lipids, flavonoids, amino and organic acids, among others) were identified in polar and no polar extracts from 17 cultivars of *Cannabis sativa* L. using the data obtained by gas chromatography–time-of-flight/mass spectrometry (GC–TOF/MS) and LC–QTOF MS/MS platforms. A number of commercial standards of cannabinoids, and terpenoids allowed confirming their identification. Relative contents of secondary metabolites (terpenoids and cannabinoids) in the same cultivars grown in greenhouse and in field were compared, and the found compositional differences between both types of grown conditions open a door for a more rational cultivation of this plant as a function of its use.

The results of these studies revealed the suitability of both LC–QTOF MS/MS and GC–MS for characterization of polar/low-polar compounds from plant samples and provided the clues for identification of their metabolites.

Sections C and D share as common denominator a relatively new clinical sample: sweat. For this reason, both sections have been discussed together. First, key contributions of the sweat–clinical metabolomics binomial were the proposal of panels of biomarkers for lung cancer detection and the quantitative determination of amino acids in sweat. Second, a method to enhance the metabolomics coverage of human sweat by GC–MS was developed. Finally, the first steps of the analytical process required by this new clinical sample are discussed: sampling —taking advantage of the multiple forms and situations by which this biofluid can be sampled— and sample preparation —mandatory step to obtain the analytical sample as required for introduction in the target analytical platform.

Sections C and D. Sweat and metabolomics: Sampling and sample preparation, and clinical applications

Sweat is mainly composed by water, but it contains several minor components including electrolytes, ammonia, urea, small molecules such as carboxylic acids and amino acids as well as more complex biomolecules such as proteolytic enzymes and antimicrobial peptides, among others [63]. The varied composition, which can be modified by certain pathologies, and the no invasive sampling of this biofluid have gained clinical interest as a potential tool for diagnostics and biomarker monitoring. As a previous step to the use of this biofluid in the clinical field, metabolomics has been used as a tool to obtain a snapshot of the composition of sweat metabolome. A method developed for analysis of human sweat by LC–QTOF MS/MS allowed identifying 41 compounds, mainly amino acids, being the most abundant compounds in this sample [64]. From this research developed by the group of the PhD student, two research lines emerged.

The first line consisted of using the platform previously proposed [65] to develop a more robust predictive model for lung cancer screening (Chapter XI) and a method for quantitation of amino acids as potential biomarkers of lung cancer in the target biofluid (Chapter VII) by LC—MS/MS. The second line consisted of developing a GC—MS platform

to elucidate sweat metabolome (Chapter X), then used to test different sample preparation protocols and samplings (Chapters VIII and XI).

The active research on omics disciplines had been previously focused on understanding the development and biology of lung cancer and identifying potential biomarkers to detect initial stages in the development of the disease [66]. Metabolomics had provided new technical approaches to enhance the capability of this discipline on biomarker discovery for diagnosis of different types of cancer. The potential of both sweat as biofluid and metabolomics for implementation in the diagnostic of lung cancer had been demonstrated in a previous study developed by the group of the PhD student [65]. Human sweat had been used as clinical sample to develop a tool for lung cancer screening. A prediction model based on a panel of sweat metabolites (80% specificity and 79% sensitivity), including amino acids, sugars, and some lipids, was built to discriminate patients with lung cancer from a control group with risk factor. These preliminary results, obtained with a unique cohort, emphasized the necessity of new studies, as that in Chapter XI, to validate the obtained results and reduce the proportion of individuals that would be subjected to confirmatory tests.

Chapter XI proposes a prediction model for lung cancer detection more robust than that obtained in the previous study. The prediction model was based on two panels of sweat metabolites in which both false negatives and false positives were reduced in such a way that 95% was the level reached for both specificity and sensitivity. The best panel providing specificity above 95% was formed by five metabolites: the monoglyceride MG(22:2), muconic, urocanic and suberic acids, and a tetrahexose. This panel provided 96.9% specificity and 83.8% sensitivity. On the other hand, the best panel providing sensitivity above 95% was composed of the metabolites included in the previous panel, in which suberic acid was replaced by nonanedioic acid (the previous panel provided 81.2% specificity and 97.3% sensitivity). The robustness of the previous biomarker panel by Calderón-Santiago *et al.* [65] was increased in Chapter XI, using new samples collected within different times (4 months and 2 years) and analyzed at different times (2012 and 2014, respectively) by different analysts, and always with the aim of discriminating lung cancer patients from smokers at risk factor.

Among 16 sweat metabolites used to discriminate lung cancer patients from control individuals, 5 of them were amino acids [65]. The composition of free amino acids in sweat is different from that in other biofluids, according to studies developed by automated

analyzers [67], thus increasing the interest in this sample for quantitative analysis of amino acids for diagnosis purposes. Also, methods based on automated analyzers had detected up to 26 amino acids in human sweat, which allow confirming this biofluid as suitable for complete profile of amino acids [67]. However, derivatization of amino acids, a common practice prior to their photometric or fluorimetric detection [68], suffers from different drawbacks, and dramatically enlarges sample preparation protocols [69]. Chapter VII affords the development of a method to quantify 23 amino acids in human sweat by LC–MS/MS. Matrix effects decreased by both dilution of sweat and clean-up effect provided by centrifugal solid-phase microextraction (c-SPμE). Amino acids behave differently among them; therefore, compromise solutions were necessary for their determination in a single analysis. The concentration of amino acids in sweat ranges between 6.20 ng/mL (for homocysteine) and 259.77 μg/mL (for serine).

In this way, Chapters XI and VII open the door to the use of no common samples as sweat in clinical studies for diagnosis of diseases as lung cancer.

The second line of the research on sweat–metabolomics was based on improving the detection coverage for identification of metabolites in this biofluid. Considering that more research on sweat composition is demanded to assess the potential of this biofluid for clinical diagnostic, and that before Chapter X only NMR [70] and LC–MS [64] had been used for sweat characterization, sweat metabolome including volatile and easy to convert into volatile compounds needed to be elucidated. Chapter X contains the development and validation of a method for analysis of human sweat by GC–TOF/MS in high resolution mode, which allowed studying a wide variety of these compounds. GC–TOF/MS allowed detecting both volatile and no volatile compounds from human sweat after applying sweat deproteination and well-known derivatization reactions. It provided information for tentative identification of 66 compounds, including amino acids, dicarboxylic acids and other interesting metabolites such as myo-inositol or urocanic acid. GC–TOF/MS appears to be the best strategy to analyze this biofluid, in terms of metabolome coverage, as compared to previous studies using NMR and LC–MS. It is worthy to distinguishing the ability of GC–MS to detect sugars, lipids and carboxylic acids, families that with previous platforms seemed to have insignificant presence, but they constitute around 20% of the whole entities detected by GC–MS. These three groups, together with amino acids, which constitute 30% of the identified compounds, are the four most remarkable families in sweat. The excellent characteristics of the developed method and the identification of

metabolites involved in key biochemical pathways emphasized the necessity of new studies, as those in Chapters VIII and IX, to set the pattern of the type of sample to be obtained depending on the metabolites of interest (polar, no polar or medium polarity compounds).

Among 66 compounds identified in Chapter X only 14 no polar compounds were detected, because most of them were of polar nature. Similar results had been found using NMR [71] and LC–MS [64]. Regarding no polar compounds, NMR analysis only allowed detection of some characteristic groups of lipids. This fact could be explained by the low concentration of lipids in the collected samples (<0.01 mM) and the low resolution of ^{1}H-NMR to discriminate lipid families. The developed LC–QTOF MS/MS analysis confirmed that the main families of detected metabolites were polar compounds, and only 4 no polar compounds (3 fatty acids and 1 sphingolipid) were detected [64].

All the studies discussed above had in common the collection of the known as passive sweat —that collected from individuals at rest. The sampling devices employed could explain the differences in sweat composition, and the lack of no polar compounds [71]. Sweating is usually stimulated by heat and/or chemical induction (for instance, using pilocarpine), but also by exercise practice at different intensity levels [72]. The first two protocols, based on temperature increase or chemical stimulation, allow collection of passive sweat, while that collected after exercise practice provides the known as active sweat, which can set differences at compositional level as compared to passive sweat.

Considering that sampling processes for this scantly known clinical sample in the above commented studies were based on passive sweat and that lipids had been scarcely detected in this sample, a method for active sweat analysis, with special emphasis on no polar compounds, was developed in Chapter VIII. A GC–TOF/MS platform allowed the tentative identification of 135 compounds. Lipids, VOCs, benzenoids and other interesting metabolites such as alkaloids and ethanolamines were identified among them more than 40 lipids. Sample preparation to obtain a representative snapshot of active sweat metabolome consisted of methoximation plus silylation after LLE with dichloromethane. Compositional differences in passive and active sweat collected from the same volunteers were found, the latter presenting an enriched concentration of some key families of compounds such as fatty acids, alcohols, carbohydrates and no proteinogenic amino acids. The differences in concentration can be explained by the sampling protocol since sweat is stimulated in a different manner prior to collection of active or passive sweat.

Focusing again on sampling, all previous studies had in common the collection of the known as passive sweat but also that it was always collected as fresh sweat, and used as such [64,70,71,73]. In most of the wide variety of fresh sweat samplers it is mandatory to wear the device for sweat production, which is a tedious process. For this reason, novel procedures for sweat collection are desirable. One alternative is collection of dry sweat, a concept that should not be confused with applications based on sampling of fresh sweat, then dried for analysis. This protocol was proposed to standardize the sampling process due to its simplicity [74]. In Chapter IX, a solid support as filter paper impregnated with 1:1 (v/v) ethanol–phosphate buffer to collect sweat components from the skin was proposed as a standardized protocol alternative to fresh sweat sampling. The collected dry sweat allowed tentative identification of 175 compounds by using a combined approach based on GC–MS and LC–MS/MS analysis. The comparison between dry and fresh sweat revealed that the former provides an improvement in the detection of low-polar compounds, while fresh sweat is more suited for detection of polar metabolites. In addition, particular families such as carnitines, sphingolipids and N-acyl-amino acids never reported in fresh sweat were detected in dry sweat. Considering the variability sources affecting the sampling of fresh sweat, collection of dry sweat can be proposed as a standardized sample from this biofluid for its use in metabolomics and future clinical studies.

In short, Chapter X open the door to the use of a GC–MS platform for sweat samples analysis in clinical studies and Chapters VIII and IX to select the type of sweat sample to be obtained depending on the metabolites of interest.

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Conclusiones

La investigación realizada y que ha dado lugar a esta Memoria de Tesis ha permitido extraer las siguientes conclusiones:

La extensa búsqueda bibliográfica, que ha propiciado las publicaciones que constituyen los Capítulos del I al III, permite una evaluación crítica del estado actual de los métodos basados en USAE para obtener componentes de plantas/alimentos y de los controvertidos efectos de los US en las enzimas y en su acción biocatalítica, pero también proporciona pautas para planificar investigación en metabolómica de plantas asistida por US.

La investigación experimental contenida en esta Memoria de Tesis se orientó al análisis metabolómico, tanto dirigido como global, en las áreas vegetal y animal. La diversidad de los estudios desarrollados incluye el efecto de la preparación de la muestra en la cobertura conseguida en la detección y el efecto favorable de los US en las reacciones enzimáticas. La variedad de los estudios realizados también abarca el efecto del muestreo y la preparación de la muestra en la eficacia de la detección de componentes del sudor, así como el estudio de los cambios en los perfiles de los metabolitos en el sudor humano recogido después de estimulación mediante diferentes procedimientos.

Las conclusiones más destacables de la investigación en metabolómica en el área vegetal son:

Capítulo IV:

- El método basado en USAEH para la obtención de la aglicona de la oleuropeína a partir oleuropeína de extractos de hojas de olivo, que requiere menos de 20 min para la hidrólisis completa, lo que permite una comparación favorable con el método tradicional basado en incubación enzimática, que requiere 2 h.

Capítulo V:

- El fraccionamiento basado en extracción líquido–líquido secuencial con extractantes de diferente polaridad mejora drásticamente la cobertura de la identificación tentativa de metabolitos en los extractos enzimáticos acuosos del *A. bisporus* (123 metabolitos tentativamente identificados).

Capítulo VI:

- Se han identificado 169 compuestos (cannabinoides, terpenoides, lípidos, flavonoides, aminoácidos y ácidos orgánicos, entre los más destacables) en extractos polares y no polares, obtenidos con la asistencia de US, de 17 cultivos de *Cannabis sativa* L. Las plataformas GC–TOF/MS y LC–QTOF MS/MS maximizan la cobertura conseguida en la detección de componentes del Cannabis, consiguiéndose identificar 46 compuestos en los extractos polares y 134 en los no polares.

En cuanto al efecto del muestreo y de la preparación de la muestra en la detección de metabolitos en sudor humano (Sección C), el uso de análisis dirigido y no dirigido permitió obtener las siguientes conclusiones:

Capítulo VII:

- El sudor es una muestra excelente para el análisis cuantitativo del perfil de aminoácidos en estudios clínicos, lo que resulta de interés teniendo en cuenta el papel biomarcador de estos compuestos. La dilución+c-SPμE ha demostrado ser decisiva para disminuir el efecto matriz del sudor humano y hacer así posible la cuantificación de aminoácidos mediante LC–MS/MS.

Capítulo VIII:

- El sudor activo, con respecto al sudor pasivo, está enriquecido en algunas familias de compuestos claves, tales como ácidos grasos, alcoholes, carbohidratos y aminoácidos no proteinogénicos. La LLE con diclorometano y posterior metoximación más sililación es la mejor opción entre los protocolos ensayados para obtener mediante GC–MS una instantánea representativa de los compuestos no polares del sudor activo.

Capítulo IX:

- El muestreo de sudor seco es una alternativa para la estandarización de la recogida de sudor. El papel impregnado con etanol–PBS en proporción 1:1 (v/v) proporciona los mejores resultados en términos de cobertura de la detección utilizando un análisis combinado mediante las plataformas GC–MS y LC–MS/MS. Se han detectado en sudor seco familias tales como carnitinas, esfingolípidos y N-acil-aminoácidos, que no se han encontrado en sudor fresco.

Los hallazgos más destacables en la mejora en cobertura metabolómica del sudor para estudios clínicos han sido los siguientes:

Capítulo X:

- La optimización de una nueva plataforma basada en GC–MS para el análisis de sudor revela la presencia de metabolitos que pueden ser de interés para el estudio de diferentes patologías. Es destacable la capacidad de la plataforma para detectar azúcares, lípidos y ácidos carboxílicos, familias cuya presencia había resultado insignificante cuando se utilizaron plataformas basadas en RMN o en LC–MS.

Capítulo XI:

- El diseño de dos paneles de cinco metabolitos que incluyen aminoácidos, ácidos dicarboxílicos de cadena corta, azúcares y algunos lípidos permite discriminar pacientes con cáncer de pulmón del grupo de control con factor de riesgo. Este nuevo modelo de predicción es más robusto que el obtenido previamente por nuestro grupo, ya que se construyó con dos tandas de muestras recogidas y analizadas con una diferencia de tiempo de dos años y por diferentes analistas.

Conclusions

The following are the conclusions from the research that constitutes this PhD-Book:

The in-depth bibliographic search (Chapters I-to-III) allows a critical evaluation of the state-of-the-art of USAE methods to obtain components from plants/foods, and the controversial effects of US on enzymes and their biocatalytic action, but also provides guidelines to plan the research on plants through metabolomics helped by US.

The experimental research in this Thesis-Book was focused on the use of both targeted and untargeted metabolomics analysis for plant and animal studies. The diversity of the developed studies includes the effect of sample preparation on detection coverage, and the favorable US effect on enzymatic reactions. The variety of the developed studies also covers the effect of both sampling and sample preparation on the efficient detection of sweat components, and the study of the changes in the metabolite profiles of human sweat collected after different sweat stimulation procedures.

The most outstanding conclusions from the research devoted to studying the metabolomics in the plant field are:

Chapter IV:

- The USAEH method to obtain oleuropein aglycon from oleuropein in olive leaf extracts requires less than 20 min for complete hydrolysis, which compares favorably with the traditional method based on enzymatic incubation, for which 2 h are necessary.

Chapter V:

- Fractionation based on sequential liquid–liquid extraction with extractants of different polarity drastically improves the tentative identification coverage of metabolites in *A. bisporus* aqueous enzymatic extracts (123 tentatively identified metabolites).

Chapter VI:

- A total of 169 compounds (cannabinoids, terpenoids, lipids, flavonoids, amino and organic acids, among others) have been identified in polar and no polar extracts obtained by US assistance from 17 cultivars of *Cannabis sativa* L. GC–

TOF/MS and LC–QTOF MS/MS maximize the detection coverage of *Cannabis* components, so 46 compounds were identified in polar extracts and 134 compounds in no polar extracts.

Concerning the effect of sampling and sample preparation on the detection of metabolites from human sweat (Section C), untargeted and targeted analysis allowed reaching the following conclusions:

Chapter VII:

- Sweat is an excellent sample for quantitative profiling analysis of amino acids in clinical studies, which is of interest taking into account the biomarker role of these compounds. Dilution+c-SPμE has proved to be mandatory to decrease the matrix effect of human sweat thus making possible absolute quantitation of amino acids by LC–MS/MS.

Chapter VIII:

- Active sweat is enriched in some key families of compounds such as fatty acids, alcohols, carbohydrates and no proteinogenic amino acids as compared with passive sweat. LLE with dichloromethane with subsequent methoxymation plus silylation is the best option among the tested protocols to obtain a representative snapshot by GC–MS of active sweat due to the detection of no polar compounds.

Chapter IX:

- Dry sweat sampling is an alternative to standardize sweat collection. Paper impregnated with 1:1 (v/v) ethanol–PBS provides the best results in terms of detection coverage by using a combined approach based on GC–MS and LC–MS/MS analysis. Particular families such as carnitines, sphingolipids and N-acyl-amino acids detected in dry sweat have not been reported in fresh sweat.

The most remarkable findings on enhancement of metabolomics coverage of sweat for clinical studies are the following:

Chapter X:

- The optimization of a new GC–MS platform for sweat analysis reveals the presence of metabolites that can be of interest for the study of different pathologies. It is worthy to remarking the ability of GC–MS to detect sugars,

lipids and carboxylic acids, families that with previous platforms (NMR and LC–MS) seemed to have insignificant presence.

Chapter XI:

- Two panels of five metabolites including amino acids, short chain dicarboxylic acids, sugars and some lipids allow discriminating patients with lung cancer from a control group with risk factor. This new prediction model is more robust than that obtained previously by our group since it was built with two batches of samples collected and analyzed with a shift time of two years and by different analysts.

Anexos

Annexes

Anexo I. Artículos de revisión sobre temas relacionados con la Tesis

Annex I. Review articles on subjects related to the Thesis

1. The 'in medium virtus' assessment of green analytical chemistry

   M.M. Delgado-Povedano, M.D. Luque de Castro

   Current Opinion in Green and Sustainable Chemistry, 2019, 19, 8–14.

   This review comes from a themed issue on Green Analytical Chemistry

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2. Ultrasound-assisted extraction and *in situ* derivatization

   M.M. Delgado-Povedano, M.D. Luque de Castro

   Journal of Chromatography A, 2013, 1296, 226–234.

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3. Ultrasound-assisted analytical emulsification–extraction

   M.M. Delgado-Povedano, M.D. Luque de Castro

   Trends in Analytical Chemistry, 2013, 45, 1–13.

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Current Opinion in Green and Sustainable Chemistry

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The 'in medium virtus' assessment of green analytical chemistry

M. M. Delgado-Povedano[1,2] and M. D. Luque de Castro[1,2]

Summary

The frantic race of analytical chemists to develop methods that fulfill the 12 principles of green analytical chemistry makes it convenient to balance the pros and cons derived from adoption of them. Thus, avoidance of sample treatment; minimization of both sample size and number of samples; development of in situ measurements, if possible; integration of analytical steps; selection of automated and miniaturized methods; avoidance of derivatization reactions; reduction of waste volumes; preference for multianalyte methods; minimization of energy required; selection of reagents from renewable sources; elimination of toxic reagents; and increase of the operator safety are aspects critically discussed in the text by proposing application of the common sense as a function of the faced situation.

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Edited by **Farid Chemat** and **Miguel de la Guardia**

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Introduction

The responsibility of analytical chemists in supporting the conclusions of the Pimentel report [1], the subsequent model of environmental analytical chemistry [2], and the concept of green chemistry [3] led to the concept of green analytical chemistry (GAC) [4], which refers to reducing or eliminating hazardous chemicals from analytical processes to improve the environmental friendliness without compromising method performance, in short to increase the use of green reagents and reduce the format of analytical methods as much as possible.

Change to green analytical reagents to propose a greener method can involve eliminating or reducing a given reagent, to replace it with other reagents that are less toxic, safer, obtained with the use of less energy or with energy from renewable sources, and easily degradable. Once these aspects, but the first, have been fulfilled as much as possible, there are others as crucial as selection of the reagent, namely, (i) subsequent steps of the analytical process involving reagents or solvents that require removal (e.g., evaporation), with the corresponding energy consumption and environmental contamination; (ii) detection that depends on the complexity of the instrument will require more or less electrical energy consumption; and (iii) waste handling that involves to reduce, as much as possible, the waste produced after analysis, ideally by recycling, by on-line decontamination, or, a less-preferred way, at-line decontamination, with as low as possible energy consumption in any case. In all of them, the necessary electrical energy should be decisive, as energy production always involves contamination—less significant in the case of renewable energies.

Reduction of the sample–reagent volumes and analytical devices has evolved from the macroscale to mesoscale, miniscale, nanoscale, and so on, in a frantic race to a scale as lower as possible. Nevertheless, is the lowest scale the best from all points of view? May a lower scale complicate the chemical and physical mechanisms involved in the process, detract for sample representativeness, precision, and/or sensitivity to limits that make the method inacceptable and the results thus obtained useless? Where is the point at which miniaturization is less important than the problems involved in reducing the size of the analytical tool?

Green analytical metrics is the discipline to assess the greenness of analytical procedures, that is, the tools to verify and quantitatively or qualitatively evaluate the greenness of each analytical step. As an example, the life cycle assessment, a 'cradle-to-grave' assessment, has been integrated by GlaxoSmithKline and AstraZeneca into their solvent assessment guides and solvent selection guides and by Pfizer for sertraline production [5] and should be taken into account by authors who put the label of 'green' to any minimal improvement of an analytical method. Other approach is the labeling with National Environmental Methods Index (NEMI) symbols (circles filled green or not if certain assumptions are fulfilled) [4] or application of the analytical Eco-Scale (classification as a function of the environmental impact) [6] as examples of no or yes application of multivariate statistical techniques, respectively. The latter is one of the most useful tools to discover and

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Review

Ultrasound-assisted extraction and *in situ* derivatization

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ABSTRACT

Ultrasound (US) is a type of energy of growing interest for analytical chemists. The favorable effect of US on mass transfer between two phases in the same or different state has been widely demonstrated as did the number of chemical reactions accelerated/improved under ultrasonic influence. The action of US on both mass transfer and chemical reaction developed in a simultaneous manner results in a synergistic effect as a consequence of the "removal" of the target extracted species by conversion into a product. Both US-assisted solid–liquid extraction and liquid–liquid extraction have been developed with *in situ* derivatization at the meso- and microscale and the advantages involved in their implementation are discussed in this article.

Contents

1. Introduction

Ultrasound (US), or sound of frequencies above human hearing spans within the range from 20 kHz to the GHz, which can be split into a power region and a diagnostic region. The former, on the low-frequency end, provides enough acoustic energy to produce cavitation which is of great interest from the analytical point of view for sample preparation. High-frequency US, endowed with low amplitude and power, does not produce cavitation and it is used for medical scanning, chemical analysis and the study of relaxation phenomena, among the most salient applications.

The typical effect of power US which supports the majority of its chemical actions is cavitation, consisting of expansion and compression cycles caused by negative and positive pressures in the

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Ultrasound-assisted analytical emulsification-extraction

M.M. Delgado-Povedano, M.D. Luque de Castro

The usefulness of ultrasound (US) for improving steps in analytical chemistry has been widely demonstrated in the past decade. One of the recent applications of US is to facilitate formation of emulsion for dramatic enhancement of the contact between surfaces of two immiscible phases. The effect is to enhance mass transfer between phases (liquid-liquid extraction) in miniaturized systems and to promote leaching of polar and non-polar components (solid-liquid extraction).

We discuss potential uses with special emphasis on the achievements and the weak points of the present research.

Keywords: Emulsification; Extraction; Immiscible phase; Leaching; Liquid-liquid extraction (LLE); Liquid-liquid microextraction (LLME); Solid-liquid extraction (SLE); Ultrasound-assisted emulsification (USAE); Ultrasound-assisted emulsification-liquid-liquid extraction (USAE-LLE); Ultrasound-assisted emulsification microextraction (USAE-ME)

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1. Introduction

Despite the vast range of frequencies of the ultrasound (US) zone, frequently divided between power US (range 20–100 kHz) and diagnostic US (within the MHz range), the former is the only range used by analytical chemists. For power US, the typical effect, which supports the majority of its chemical actions, is cavitation, a phenomenon consisting of expansion and compression cycles caused by negative and positive pressures in the liquid subjected to US. The expansion cycle pulls molecules away from one another and creates bubbles or cavities in the liquid, which grow and collapse; thus constituting the cavitation phenomenon. There are two main types of acoustic cavitation: "transient" and "stable". The mechanism by which acoustic emulsification occurs is not fully understood, despite this phenomenon being reported for the first time in 1927 [1], but cavitation is thought to be responsible for most sonochemical effects, including acoustic emulsification of oil droplets [2]. The degree of acoustic emulsification depends on certain parameters, notably US power and chemical composition of the system. Beyond optimal conditions, US may cause the opposite effects (viz coagulation and precipitation) [3]. The advantages of US for emulsification, compared to mechanical emulsion, include lower energy consumption, use of less surfactant and production of a more homogeneous, finer emulsion.

There are currently two main types of US laboratory equipment available in the market for analytical laboratories (i.e. cleaning baths and probes).

The US cleaning bath (designed for cleaning glassware and degassing) is the most affordable and inexpensive US laboratory device available in the market. This is why it is omnipresent in any laboratory and most analytical chemists use it for analytical tasks that require more precise US equipment. The main limitations of US cleaning baths, which are the sources of their irreproducibility, are the decline of power with time and the lack of uniformity in the transmission of US. In addition, not all US cleaning baths operate at the same frequency. This may affect the results and be a problem when attempting to reproduce previously reported results.

US probes, also known as sonotrodes, are not subjected to any exhaustion restrictions, so they are much more suitable for use in analytical laboratories than US cleaning baths, but they are also more expensive and less robust. In addition, the power and duty cycle [i.e. the fraction of time that they are working (e.g., a fraction of s per s)] can be programmed, as required. They always work at fixed frequency (usually 20 kHz or 40 kHz). When

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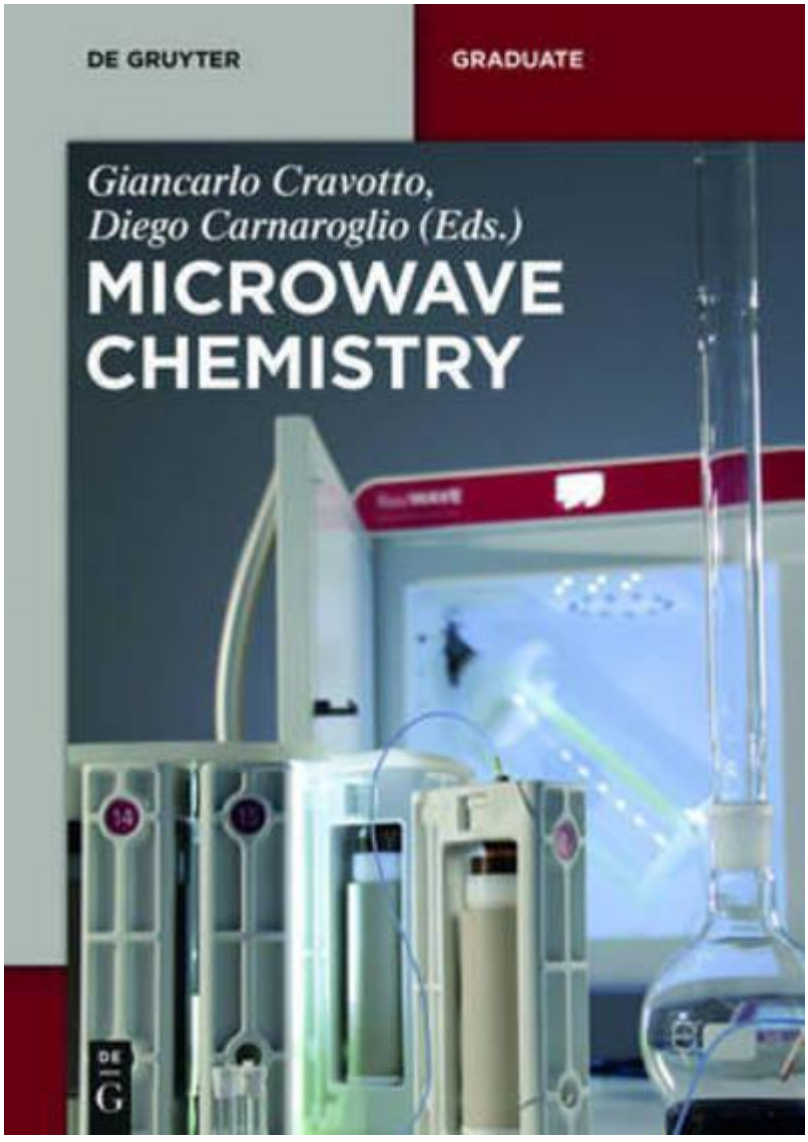
Anexo II. Capítulo de libro sobre un tema relacionado con la Tesis

Annex II. Book chapter on a subject related to the Thesis


DE GRUYTER
GRADUATE
Giancarlo Cravotto,
Diego Carnaroglio (Eds.)
MICROWAVE
CHEMISTRY

María M. Delgado-Povedano and María D. Luque de Castro

22 Microwaves in the omics field

Abbreviations list:

ELISA, enzyme-linked immunosorbent assay
emPCR, emulsion polymerase chain reaction
ESI-MS/MS, electrospray ionization-tandem mass spectrometry
FISH, fluorescence *in situ* hybridization
FF-PET, formalin-fixed, paraffin-embedded tissue
GLP, good laboratory practice
ICATt, isotope-coded affinity tags
ICP, inductively coupled plasma
imFASP, filter-aided sample preparation
iTRAQt, isobaric tags for relative and absolute quantitation
LC, liquid chromatography
LLE, liquid–liquid extraction
MAAH, microwave-assisted acid hydrolysis
MAE, microwave-assisted extraction
MALDI, matrix-assisted laser desorption ionization
MAMEF, microwave-accelerated metal-enhanced fluorescence
MT-MEC, microwave-triggered metal-enhanced chemiluminescence
MW, microwave(s)
NGS, next-generation sequencing
PCR, polymerase chain reaction
QTOF, quadrupole time of flight
RCA, rolling circle amplification
TFA, trifluroacetic acid

22.1 Introduction

Omics is at present a magic suffix from the Latin *ome*, which means mass or massive and refers to the enormous number of analytical data produced and required to obtain the information pursued by the so-called omics disciplines. Thus, an analytical process must be used to obtain the data required in an omics study. One or several steps of the analytical process can be assisted by microwave (MW) energy to accelerate, improve, or allow the acquisition of target analytical data.

https://doi.org/10.1515/9783110479935-022

Anexo III. Experto en Docencia Universitaria, con título propio de la Universidad de Córdoba

Annex III. Expert in University Teaching, graduating from the University of Córdoba

La Universidad de Córdoba

y en su nombre el Rector

De acuerdo con el Reglamento de Estudios Propios de esta Universidad,
expide en favor de

Doña María del Mar Delgado Povedano

con D.N.I. 15.404.645-G,
el presente título propio de

Experto en Docencia Universitaria

con la calificación de APTO

al haber superado el Plan de Estudios que figura al dorso, cursado durante los cursos 2016/17
y 2017/18 y organizado por el Vicerrectorado de Estudios de Posgrado y Formación Continua.

Dado en Córdoba, a 8 de noviembre de 2018

La Secretaria General,	El Rector,	El Director Académico,
María del Carmen Balbuena Torezano	José Carlos Gómez Villamandos	Juan de Dios Torralbo Caballero

Este título tiene carácter de propio, según lo dispuesto en el art. 34.1 de la Ley Orgánica 6/2001 y en la Disposición adicional undécima del Real Decreto 1393/2007.

Nº Registro 202221

Denominación: **Experto en Docencia Universitaria**

ASIGNATURAS	CRÉDITOS
Fase I. Formación	**9**
- Metodología Docente	1
- Evaluación	1
- Coordinación y trabajo en equipo	1
- Orientación y tutoría	1
- Competencias personales, interpersonales y comunicativas	1
- Responsabilidad y compromiso social universitarios	1
- Planificación docente	1
- Marco normativo y gestión universitaria	1
- Innovación e investigación docente	1
Fase II. Práctica docente mentorizada	**3**
Fase III. Innovación tutelada	**6**

TOTAL DE CRÉDITOS ECTS: 18 (1 ECTS = 25 horas)
Horas Presenciales: 180 horas. Trabajo del Estudiante: 270 horas.

Anexo IV. Artículo de investigación derivado de un Proyecto del Plan de Innovación y Buenas Prácticas Docentes

Annex IV. Research article from a Project of the Innovation Plan and Good Teaching Practices

REVISTA DE INNOVACIÓN Y BUENAS PRÁCTICAS DOCENTES

DESARROLLO DE UN MÉTODO PARA LA EVALUACIÓN DE LA ADQUISICIÓN DE COMPETENCIAS Y SU COMPARACIÓN CON EL SISTEMA TRADICIONAL DE EVALUACIÓN EN ASIGNATURAS DEL GRADO DE QUÍMICA

Manuel Blázquez Ruiz, Feliciano Priego Capote, Guadalupe Sánchez Obrero, Mª del Mar Delgado Povedano

Resumen/Abstract

Un aspecto diferencial de la enseñanza de Grado en el Espacio Europeo de Enseñanza Superior (EEES), recogido en el Marco Español de Cualificaciones (MECES), es la definición de programas formativos que implican conocimientos y competencias. En este trabajo se han preparado rúbricas para evaluar las competencias a adquirir por el alumnado en dos asignaturas del Grado en Química. Las rúbricas diseñadas permiten dividir el conjunto de competencias en elementos diferenciados que se pueden evaluar diseñando y seleccionando actividades apropiadas. En esta herramienta se definen indicadores escalados para determinar el nivel alcanzado en cada competencia. Los resultados obtenidos con la aplicación de las rúbricas han sido correlacionados con los obtenidos en la evaluación habitual. Se propone así un método de trabajo flexible y dinámico para mejorar el proceso de evaluación.

Palabras clave/Keywords

Competencias, evaluación, rúbricas, sistema tradicional, grado de química.

Texto completo:

PDF

DOI: https://doi.org/10.21071/ripadoc.v7i0.11667

Enlaces refback

- No hay ningún enlace refback.

DESARROLLO DE UN MÉTODO PARA LA EVALUACIÓN DE LA ADQUISICIÓN DE COMPETENCIAS Y SU COMPARACIÓN CON EL SISTEMA TRADICIONAL DE EVALUACIÓN EN ASIGNATURAS DEL GRADO DE QUÍMICA

DEVELOPMENT OF A METHOD FOR THE EVALUATION OF COMPTENCES ACQUISITION AND ITS COMPARISON WITH THE TRADICIONAL EVALUATION SYSTEM IN SUBJECTS OF CHEMISTRY DEGREE

Manuel Blázquez Ruiz*, Feliciano Priego Capote**,
Guadalupe Sánchez Obrero, Mª del Mar Delgado Povedano
*qf1blrum@uco.es, **q72prcaf@uco.es

Received: 29/06/2018 Accepted: 30/10/2018

Resumen

Un aspecto diferencial de la enseñanza de Grado en el Espacio Europeo de Enseñanza Superior (EEES), recogido en el Marco Español de Cualificaciones (MECES), es la definición de programas formativos que implican conocimientos y competencias. En este trabajo se han preparado rúbricas para evaluar las competencias a adquirir por el alumnado en dos asignaturas del Grado en Química. Las rúbricas diseñadas permiten dividir el conjunto de competencias en elementos diferenciados que se pueden evaluar diseñando y seleccionando actividades apropiadas. En esta herramienta se definen indicadores escalados para determinar el nivel alcanzado en cada competencia. Los resultados obtenidos con la aplicación de las rúbricas han sido correlacionados con los obtenidos en la evaluación habitual. Se propone así un método de trabajo flexible y dinámico para mejorar el proceso de evaluación.

Palabras clave: Competencias, evaluación, rúbricas, sistema tradicional, grado de química

Abstract

A differential aspect of the teaching of Degree in the European Higher Education Area (EHEA), included in the Spanish Qualifications Framework (MECES), is the definition of training programs that involve knowledge and skills. In this paper, rubrics have been prepared to evaluate the competences to be acquired by the students in two subjects of the Degree in Chemistry. The designed rubrics allow dividing the set of competences into differentiated elements that can be evaluated by designing and selecting appropriate activities. In this tool, scaled indicators are defined to determine the level reached in each competence. The results obtained with the application of the rubrics has been correlated with those obtained in the usual evaluation. A work method that is flexible and dynamic to improve the evaluation process is proposed.

Keywords: Competencies, evaluation, rubrics, traditional system, chemistry degree

1. INTRODUCCIÓN

Un aspecto diferencial de la enseñanza de Grado en el marco del Espacio Europeo de Enseñanza Superior (EEES) es la definición de un programa formativo que implica conocimiento y competencias frente a la definición única de conocimientos específicos como se estructuraba en el plan de estudios de Licenciatura en nuestro sistema nacional.

Por ello, en el Grado es importante tomar conciencia de la importancia que en el nuevo paradigma docente tienen las competencias, en paralelo con los conocimientos, que va a adquirir el estudiante en las diferentes asignaturas de los cursos y del propio Grado. Conocer el nivel de formación que el alumnado adquiere en el proceso formativo, y en particular, durante el desarrollo de las actividades docentes es crucial.

A esto hay que añadir que en la estructura del Grado se distinguen tres elementos importantes: el bloque de asignaturas básicas, el bloque nuclear (asignaturas troncales y obligatorias) y el bloque de asignaturas optativas. Dentro de estos bloques se configuran las materias y las asignaturas del plan de estudios con los contenidos específicos para que el estudiante adquiera una formación básica, fundamental y de carácter generalista.

La evaluación de los conocimientos adquiridos y la formación del estudiante siempre suscitan debate en relación con la tipología de materias, tipología docente, tipo de prueba, ejercicios, grado de experimentación, etc. En este sentido, los planes de estudios de Grado, y su definición a través de un documento Verifica, según establece el RD 1393/2007 de ordenación de las Enseñanzas Universitarias, introduce características novedosas que deben ser tenidas en cuenta. Entre estas características, se contempla la citada conjunción de conocimientos y competencias como elemento esencial, y otras como la movilidad, el reconocimiento y transferencia, las prácticas externas y el trabajo fin de grado, que sin duda permiten una mayor flexibilidad, portabilidad y equivalencia con estudios desarrollados en otros escenarios nacionales e internacionales, académicos y profesionales.

Para alcanzar las competencias que establece el Marco Español de Cualificaciones de Enseñanza Superior (MECES) en su nivel dos, correspondiente al Grado, es muy importante la coordinación horizontal y vertical en el desarrollo de los programas formativos y en la evaluación de estos. Todo ello tutelado por un sistema de garantía de calidad que permita una mejora continua.

UNIVERSIDAD D CÓRDOBA

Dª. Julieta Mérida García, Vicerrectora de Posgrado e Innovación Docente de la Universidad de Córdoba

INFORMA:

Que, D./Dª. MARIA DEL MAR DELGADO POVEDANO, con D.N.I. 15404645G, ha participado en los siguientes Proyectos de Mejora de la Calidad Docente convocados por la Universidad de Córdoba.

Convocatoria	Participación	Número Proyecto	Titulo
2017/2018	COLABORADOR/A	2017-2-2002	DESARROLLO DE UN MÉTODO PARA LA EVALUACIÓN DE LA ADQUISICIÓN DE COMPETENCIAS Y SU COMPARACIÓN CON EL SISTEMA TRADICIONAL DE EVALUACIÓN EN ASIGNATURAS DEL GRADO DE QUÍMICA

Y para que conste y surta los efectos oportunos, expido el presente informe, en Córdoba a **martes, 24 de septiembre de 2019**

LA VICERRECTORA DE POSGRADO E INNOVACIÓN DOCENTE

Anexo V. Codirección del Trabajo Fin de Máster (TFM) de Francisco Martín Loro

Annex V. Co-supervision of the Master´s Thesis of Francisco Martín Loro

1. Título TFM: La orina como muestra clínica para la búsqueda de biomarcadores metabolómicos de cáncer de próstata sustitutivos del PSA

   Nombre y apellidos: Francisco Martín Loro

   Tutores: María Dolores Luque de Castro y María del Mar Delgado Povedano

   Calificación: 9.4

UNIVERSIDAD Ð CÓRDOBA

Julieta Mérida García, Vicerrectora de Posgrado e Innovación Docente y Directora del Instituto de Estudios de Posgrado de la Universidad de Córdoba

Certifica:

Que, Delgado Povedano, María del Mar, con DNI 15404645G

· Ha sido Directora de la asignatura Trabajo Fin de Máster, incluida en el plan de estudios de Máster Universitario en Biotecnología, en el curso 2017/2018, con el título: La orina como muestra clínica para la búsqueda de biomarcadores metabolómicos de cáncer de próstata sustitutivos del PSA, elaborado por Francisco Martín Loro con la calificación Sobresaliente

Para que conste, a los efectos oportunos, a petición del interesado/a, firmo la presente en CÓRDOBA a 14 de marzo del 2019

Código Seguro de Verificación	VFBSI2KYBEVUY6EDGWSFY475HE	**Fecha y Hora**	15/03/2019 14:47:55
Normativa	Este documento incorpora firma electrónica reconocida de acuerdo a la ley 59/2003, 19 de diciembre, de firma electrónica		
Firmado por	JULIETA MERIDA GARCIA		
Url de verificación	https://sede.uco.es/verifirma/	**Página**	1/1

UNIVERSIDAD DE CÓRDOBA

INSTITUTO DE ESTUDIOS DE POSTGRADO

MÁSTER EN BIOTECNOLOGÍA

Trabajo Fin de Máster

La orina como muestra clínica para la búsqueda de biomarcadores metabolómicos de cáncer de próstata sustitutivos del PSA

Francisco Martín Loro
Grupo de investigación FQM-227
Departamento de Química Analítica

Directora: María Dolores Luque de Castro

Codirector: María del Mar Delgado Povedano

Córdoba, 09/2018

1

Anexo VI. Codirección de los Trabajos Fin de Grado (TFG) de Ismael Ruiz González, de Eva Natalia Cárdenas Soria y de Elvira Cruces Vera

Annex VI. Co-supervision of the Final Degree Projects of Ismael Ruiz González, Eva Natalia Cárdenas Soria and Elvira Cruces Vera

1. Título TFG: Caracterización de extractos fenólicos de hoja de olivo para la preparación de concentrados purificados con interés nutricional

   Código TFG: BQ18-41-QAN

   Nombre y apellidos: Ismael Ruiz González

   Tutores: Feliciano Priego Capote y María del Mar Delgado Povedano

   Calificación: 9.4

2. Título TFG: Comparación del espectrómetro de diodos en fila y el de masas conectados a un cromatógrafo de líquidos para la cuantificación en orina de metabolitos biomarcadores de cáncer de próstata

   Código TFG: BQ17-44-QAN

   Nombre y apellidos: Eva Natalia Cárdenas Soria

   Tutores: María Dolores Luque de Castro y María del Mar Delgado Povedano

   Calificación: 8.0

3. Título TFG: Comparación del perfil metabólico de los componentes del sudor en niños, adultos y ancianos

   Código TFG: BQ16–41–QAN

   Nombre y apellidos: Elvira Cruces Vera

   Tutores: María Dolores Luque de Castro y María del Mar Delgado Povedano

   Calificación: 9.0

UNIVERSIDAD
D
CÓRDOBA

Facultad de Ciencias

MARTA ROSEL PÉREZ MORALES, SECRETARIA DE LA FACULTAD DE CIENCIAS DE LA UNIVERSIDAD DE CÓRDOBA,

CERTIFICA:

Que **Dª. Mª DEL MAR DELGADO POVEDANO,** con D.N.I.: 15404645G, ha sido tutora de los siguientes Trabajos de Fin de Grado:

- BQ18-41-QAN "CARACTERIZACIÓN DE EXTRACTOS FENÓLICOS DE HOJA DE OLIVO PARA LA PREPARACIÓN DE CONCENTRADOS PURIFICADOS CON INTERÉS NUTRICIONAL" Dicho trabajo fue defendido en la convocatoria de julio del curso 2018/2019 y obtuvo la calificación de 9,4 Sobresaliente.

- BQ17-44-QAN "COMPARACIÓN DEL ESPECTÓMETRO DE DIODOS EN FILA Y EL DE MASAS CONECTADOS A UN CROMATÓGROFO DE LÍQUIDOS PARA LA CUANTIFICACIÓN EN ORINA DE METABOLITOS BIOMARCADORES DE CÁNCER DE PRÓSTATA" Dicho trabajo fue defendido en la convocatoria de septiembre del curso 2017/2018 y obtuvo la calificación de 8 Notable.

Y para que conste y surta los efectos oportunos, firmo el presente en Córdoba, a 12 de septiembre de 2019.

Código Seguro de Verificación	VZ6JXWKPLEWGG6N47XSTKWNYTE	Fecha y Hora	12/09/2019 13:46:45
Normativa	Este documento incorpora firma electrónica reconocida de acuerdo a la ley 59/2003, 19 de diciembre, de firma electrónica		
Firmado por	MARTA ROSEL PEREZ MORALES		
Url de verificación	https://sede.uco.es/verifirma/	Página	1/1

UNIVERSIDAD DE CÓRDOBA

Facultad de Ciencias

Grado en Bioquímica

Trabajo Fin de Grado

Caracterización de extractos fenólicos de hoja de olivo para la preparación de concentrados purificados con interés nutricional

Código del TFG: **BQ18-41-QAN** Tipología: **Iniciación a la Investigación**

Autor: Ismael Ruiz González

UNIVERSIDAD DE CÓRDOBA

Facultad de Ciencias

Grado Bioquímica

Trabajo Fin de Grado

Comparación del espectrómetro de diodos en fila y el de masas conectados a un cromatógrafo de líquidos para la cuantificación en orina de metabolitos biomarcadores de cáncer de próstata

Código del TFG: BQ17-44-QAN
Tipología: Iniciación a la investigación

Autor: EVA NATALIA CÁRDENAS SORIA

07/09/2018

Department of Analytical Chemistry
Marie Curie Annex Building
Campus Universitario de Rabanales
University of Córdoba
14071 Córdoba (Spain)
Phone and Fax: +34 957 218615

MARÍA DOLORES LUQUE DE CASTRO, CATEDRÁTICA EMÉRITA DE QUÍMICA ANALÍTICA DE LA UNIVERSIDAD DE CÓRDOBA, COMO SUPERVISORA DEL TRABAJO FIN DE GRADO (TFG) DE LA ALUMNA DE CUARTO CURSO DEL GRADO DE BIOQUÍMICA ELVIRA CRUCES VERA (COMPARACIÓN DEL PERFIL METABOLÓMICO DE LOS COMPONENTES DEL SUDOR EN NIÑOS, ADULTOS Y ANCIANOS, —CÓDIGO DEL TFG BQ16–41-QAN),

Certifica: Que la licenciada **María del Mar Delgado Povedano**, actualmente finalizando su Tesis Doctoral, que presentará, expondrá y defenderá antes de final de 2019, co-supervisó en su día el citado TFG, del que no tiene un certificado oficial al no formar parte en ese momento del PID del Departamento.

Y para que conste donde proceda a instancias de la interesada, firmo el presente certificado en Córdoba, a 5 de septiembre de 2019.

Fdo.: M. D. Luque de Castro

UNIVERSIDAD DE CÓRDOBA

Facultad de Ciencias

Grado de Bioquímica

Trabajo Fin de Grado

Comparación del perfil metabólico de los componentes del sudor en niños, adultos y ancianos

Código del TFG: **BQ16 – 41 – QAN**
Tipología: **Iniciación a la investigación**

Autor: ELVIRA CRUCES VERA

4/7/17

Anexo VII. Comunicaciones orales y en carteles en congresos y en reuniones nacionales de química analítica

Annex VII. Oral and poster communications in national analytical chemistry meetings

1. **Metabolomics analysis of dry sweat by gas chromatography–time of flight/mass spectrometry and liquid chromatography–quadrupole time of flight tandem mass spectrometry in high resolution mode**

   M.M. Delgado-Povedano, L.S. Castillo-Peinado, M. Calderón-Santiago, M.D. Luque de Castro, F. Priego Capote

   XXII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

   Valladolid, 2019

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Nacional

---

2. **Metabolomics analysis of human sweat by gas chromatography–time of flight/mass spectrometry in high resolution mode**

   M.M. Delgado-Povedano, M. Calderón-Santiago, M.D. Luque de Castro, F. Priego Capote

   XXII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA
   Valladolid, 2019

   Tipo de evento: Póster en Congreso **Ámbito:** Nacional

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3. **Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea Europaea* L.) leaf extracts**

   M.M. Delgado-Povedano, M.D. Luque de Castro, F. Priego Capote
   XV REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA
   Almería, 2016

   Tipo de evento: Póster en Congreso **Ámbito:** Regional

SEQA 2019

XXII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

VALLADOLID 17, 18 Y 19 DE JULIO

LIBRO DE RESÚMENES

SEQA Sociedad Española de Química Analítica

UVa

NBO-O08

METABOLOMICS ANALYSIS OF DRY SWEAT BY GAS CHROMATOGRAPHY–TIME OF FLIGHT/MASS SPECTROMETRY AND LIQUID CHROMATOGRAPHY–QUADRUPOLE TIME OF FLIGHT TANDEM MASS SPECTROMETRY IN HIGH RESOLUTION MODE

A. María del Mar Delgado-Povedano[a,b,c,d], B. Laura S. Castillo-Peinado[a,b,c,d], C. Mónica Calderón-Santiago[a,b,c,d], D. María Dolores Luque de Castro[a,b,c,d], E. Feliciano Priego-Capote[a,b,c,d]

[a]Department of Analytical Chemistry, University of Córdoba, Annex Marie Curie Building, Campus of Rabanales, E-14071, Córdoba, email: q72prcaf@uco.es
[b]ceiA3 Agroalimentary Excellence Campus, University of Córdoba, Avd. Medina Azahara, 5, E-14071, Córdoba
[c]Maimónides Institute of Biomedical Research (IMIBIC), Reina Sofía University Hospital, Av. Menéndez Pidal, E-14004, Córdoba
[d]CIBER Fragilidad y Envejecimiento Saludable (CIBERfes), Instituto de Salud Carlos III, Monforte de Lemos 3-5, E-28029, Madrid

The popularity of sweat as clinical sample in metabolomics studies is increasing as this biofluid is non-invasively collected and its composition is influenced by several illnesses. There is a lack of standardized strategies for sampling human sweat. Most studies on this sample have been performed with fresh sweat collected after stimulation. A promising and simple alternative is sampling dry sweat by a solid support impregnated with a suited solvent. This research was aimed at comparing the metabolomics coverage provided by dry sweat collected by two solid supports (gauzes and filter papers) impregnated with three different solvents. The dissolved dry sweat was analyzed by a dual approach: LC–MS/MS and GC–MS. Among the tested sampling strategies, filter paper impregnated with 1:1 (v/v) ethanol–phosphate buffer resulted the combination providing the highest metabolomics coverage (tentative identification of one hundred fifty-six compounds). Dry and fresh sweat were compared by using pools from the same individuals to evaluate compositional differences. Families of metabolites such as carnitines, sphingolipids and N-acyl-amino acids, among others, were exclusively identified in dry sweat. Comparison of both sweat samples allowed concluding that dry sweat is more suited for analysis of low polar metabolites, while fresh sweat is better for polar compounds. As the majority of the identified metabolites are involved in key biochemical pathways, this study opens promising possibilities to the use of dry sweat as a metabolomics source of markers for specific disorders. Sampling of dry sweat could provide a standardized approach for collection of this biofluid, thus overcoming the variability limitations of fresh sweat.

NBO-P14

METABOLOMICS ANALYSIS OF HUMAN SWEAT BY GAS CHROMATOGRAPHY–TIME OF FLIGHT/MASS SPECTROMETRY IN HIGH RESOLUTION MODE

B. María del Mar Delgado-Povedano[a,b,c,d], B. Mónica Calderón-Santiago[a,b,c,d], C. María Dolores Luque de Castro[a,b,c,d], D. Feliciano Priego-Capote[a,b,c,d]

[a]Department of Analytical Chemistry, University of Córdoba, Annex Marie Curie Building, Campus of Rabanales, E-14071, Córdoba, email: q82depom@uco.es
[b]ceiA3 Agroalimentary Excellence Campus, University of Córdoba, Avd. Medina Azahara, 5, E-14071, Córdoba
[c]Maimónides Institute of Biomedical Research (IMIBIC), Reina Sofía University Hospital, Av. Menéndez Pidal, E-14004, Córdoba
[d]CIBER Fragilidad y Envejecimiento Saludable (CIBERfes), Instituto de Salud Carlos III, Monforte de Lemos 3-5, E-28029, Madrid

Human sweat is a promising biofluid to be implemented in clinical metabolomics studies by virtue of its no invasive sampling and varied composition, which is modified by several pathologies. In fact, sweat has been recently used in metabolomics studies to discriminate between lung cancer patients and risk factor individuals (mainly smokers). Elucidation of sweat metabolome has so far been achieved using three analytical platforms (NMR, LC–MS and GC–MS). One hundred and three metabolites have been detected by combination of the three platforms, being GC–MS the approach providing the highest coverage, detecting more than 60% of them. Nevertheless, these previous studies did not focus attention on non-polar metabolites, presumably a minor fraction owing to the aqueous nature of sweat. The detection of non-polar compounds in sweat would increase the interest of sweat for clinical studies, especially in dealing with biomarkers search. With these premises, a method for metabolomics analysis of human sweat by gas chromatography–time of flight/mass spectrometry (GC–TOF/MS) in high resolution mode has been developed to expand the detection of compounds, with special emphasis on non-polar compounds. Different sample preparation strategies were tested to check their influence on the metabolomics profile of sweat. The performance of three extractants with different polarity (ethyl acetate, dichloromethane and hexane) and a deproteinization protocol were compared by using, in all cases, a dual approach: (1) application of a derivatization protocol prior to GC–MS and, (2) direct injection to detect volatile organic compounds. One hundred forty-three compounds were tentatively identified by high resolution MS. Mostly lipids, but also amino acids, benzenoids and other interesting metabolites, were identified. It is worthy to distinguishing the ability of the GC–MS protocol to detect lipids, family that represented around 32% of the compounds detected by the GC–MS platform. Among the compared protocols, methyoximation plus silylation after liquid–liquid extraction with dichloromethane was the most suited option to obtain a representative snapshot of sweat metabolome. Because of the involvement of most of the identified metabolites in key biochemical pathways, this study opens new possibilities to the use of human sweat in the search for lung cancer biomarkers.

XV REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

Almería, 30 de junio y 1 de julio de 2016

GRUPO REGIONAL ANDALUZ
SOCIEDAD ESPAÑOLA DE
QUÍMICA ANALÍTICA

UNIVERSIDAD DE ALMERÍA

ALI-11

SELECTIVE ULTRASOUND-ENHANCED ENZYMATIC HYDROLYSIS OF OLEUROPEIN TO ITS AGLYCON IN OLIVE (OLEA EUROPAEA L.) LEAF EXTRACTS

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Olive leaves, a residue generated in the production of virgin olive oil, are characterized by a high phenolic content. The most abundant compound in olive leaves is oleuropein. The aglycon form of oleuropein, obtained by enzymatic hydrolysis, has been widely studied because of its proved pharmacological effects. A preliminary study was carried out to select the most suitable hydrolase among the three more frequently used in the literature. *β*-glucosidase from *Aspergillus niger* was selected as the enzyme with the highest catalytic activity. Then, the applicability of ultrasound (US) energy to accelerate this enzymatic hydrolysis was tested with the aim of developing a method to obtain oleuropein aglycon from oleuropein in olive leaf extracts in a time as short as possible and with the minimum production of other compounds. A Box-Behnken design (BBD) was used to optimize the US variables duty cycle, amplitude, and cycle time for maximum acceleration of the hydrolysis of oleuropein standard, and the optimum US conditions for achieving maximum yield of oleuropein aglycon were 0.5 s s^{-1} duty cycle, 50% amplitude and 45 s cycle. After multivariate optimization of the variables involved in the USAEH method, a kinetics study showed that the hydrolysis reaction of oleuropein was complete in only 18.75 min. A drastic shortening of the time required for complete hydrolysis was achieved as compared with a traditional method based on enzymatic incubation, which requires 2 h. US–assisted enzymatic hydrolysis of oleuropein was used as sample preparation strategy prior to quantitative analysis of oleuropein and its aglycon in olive leaves by LC–MS/MS with selected reaction monitoring. The method was applied to obtain oleuropein aglycon from commercial and laboratory extracts obtained from olive leaves, which may be tested in pharmacological applications supported on the bioactivity of oleuropein aglycon.

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Anexo VIII. Comunicaciones orales y en carteles en congresos y reuniones de investigadores jóvenes

Annex VIII. Oral and poster communications in young investigators meetings

1. **Dry sweat as sample for metabolomics analysis**

   M.M. Delgado-Povedano, L.S. Castillo-Peinado, M. Calderón-Santiago, M.D. Luque de Castro, F. Priego-Capote

   10th IMIBIC YOUNG INVESTIGATORS MEETING

   Córdoba, 2019

   Tipo de evento: Póster en Congreso **Ámbito:** Internacional

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2. **Characterization of human dry sweat metabolome by gas chromatography and liquid chromatography coupled to mass spectrometry and evaluation of sampling protocols**

   M.M. Delgado-Povedano, L.S. Castillo-Peinado, M. Calderón-Santiago, M.D. Luque de Castro, F. Priego-Capote

   9th IMIBIC YOUNG INVESTIGATORS MEETING

   Córdoba, 2018

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Internacional

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3. **Metabolomics analysis of human sweat by gas chromatography–time of flight/mass spectrometry**

   M.M. Delgado-Povedano, M. Calderón-Santiago, B. Jurado-Gámez, M.D. Luque de Castro, F. Priego-Capote

   8th IMIBIC YOUNG INVESTIGATORS MEETING

   Córdoba, 2017

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Internacional

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4. **Identificación tentativa de la composición de extractos enzimáticos acuosos de Agaricus Bisporus con actividad antiviral contra el VHC: Un estudio mediante LC-MS/MS de alta resolución**

   M.M. Delgado-Povedano, F. Priego-Capote, M.D. Luque de Castro

   V CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN DE LA UNIVERSIDAD DE CÓRDOBA

   Córdoba, 2016

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Regional

5. **Recent advances in human sweat metabolomics for lung cancer screening**

   M. Calderón-Santiago, MM. Delgado-Povedano, B. Jurado-Gámez, F. Priego-Capote, M.D. Luque de Castro

   7th IMIBIC YOUNG INVESTIGATORS MEETING

   Córdoba, 2016

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Internacional

6. **Advances in the search for biomarkers to develop a screening tool for the diagnosis of lung cancer**

   M.M. Delgado-Povedano, M. Calderón-Santiago, B. Jurado-Gámez, F. Priego-Capote, M.D. Luque de Castro

   6th IMIBIC YOUNG RESEARCHERS´ MEETING

   Córdoba, 2015

   Tipo de evento: Póster en Congreso **Ámbito:** Internacional

7. **Análisis cuantitativo del perfil de aminoácidos en sudor humano mediante cromatografía líquida-espectrometría de masas de triple cuadrupolo**

M.M. Delgado-Povedano, M. Calderón-Santiago, F. Priego-Capote, M.D. Luque de Castro

IV CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN DE LA UNIVERSIDAD DE CÓRDOBA

III CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN EN AGROALIMENTACIÓN

Córdoba, 2014

Tipo de evento: Comunicación oral en Congreso **Ámbito:** Regional

10TH IMIBIC YOUNG INVESTIGATORS MEETING

IMIBIC BUILDING
CONFERENCE ROOM
CÓRDOBA, 16-17 MAY, 2019

ABSTRACT BOOK

P3. Dry sweat as sample for metabolomics analysis

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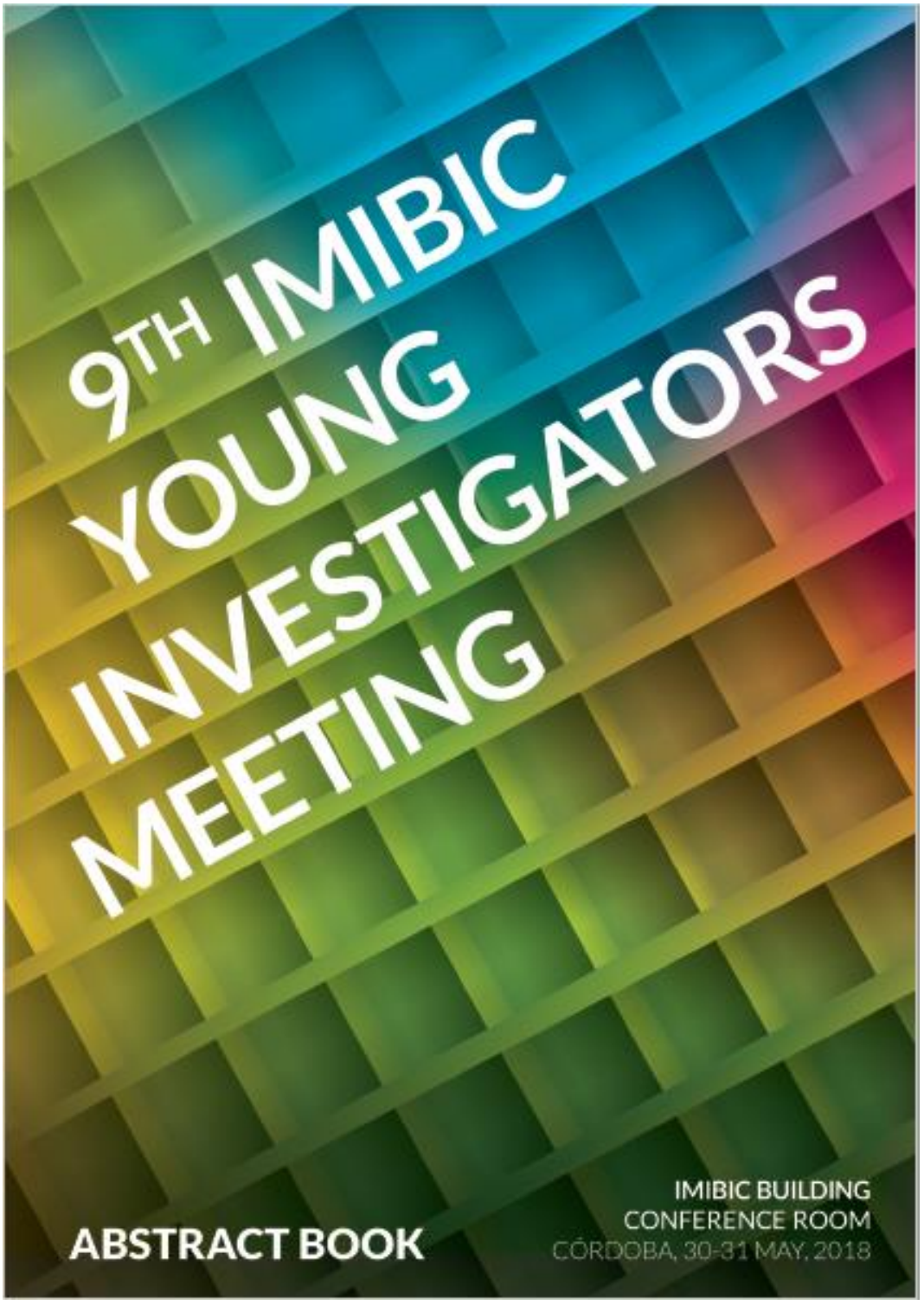
Scientific Program: Cancer (Oncology and Oncohematology)
Keywords: Dry sweat, fresh sweat, metabolomics, sampling, mass spectrometry, lung cancer

Abstract:

Sweat is gaining popularity in clinical metabolomics as this biofluid is non-invasively sampled and its composition is modified by several pathologies. In fact, human sweat has been recently used in metabolomic studies to discriminate between lung cancer patients and risk factor individuals. There is a lack of standardized strategies for collection of human sweat. Most studies have been carried out with fresh sweat collected after stimulation. A promising and simple alternative is sampling dry sweat by a solid support impregnated with a suited solvent. This research was aimed at comparing the metabolomics coverage provided by dry sweat collected by two solid supports (gauzes and filter papers) impregnated with different solvents. The dissolved dry sweat was analyzed by a dual approach: GC–MS and LC–MS/MS. Among the tested sampling strategies, filter paper impregnated with 1:1 (v/v) ethanol–phosphate buffer resulted the combination providing the highest metabolomics coverage (tentative identification of 175 compounds). Dry and fresh sweat were compared by using pools from the same individuals to evaluate compositional differences. Families of metabolites such as carnitines, sphingolipids and N-acyl-amino acids, among others, were exclusively identified in dry sweat. Comparison of both samples allowed concluding that dry sweat is better for analysis of low polar metabolites and fresh sweat is more suited for polar compounds. As most of the identified metabolites are involved in key biochemical pathways, this study opens interesting possibilities to the use of dry sweat as a source of metabolite markers for specific disorders such as cancer. In fact, N-acetyl-amino acids such as acetylhistidine had been previously found in urine from prostate cancer patients at high-significantly lower concentration than in healthy individuals. In conclusion, sampling of dry sweat could provide a standardized approach for collection of this biofluid, thus overcoming the variability limitations of fresh sweat.


9TH IMIBIC
YOUNG
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MEETING
ABSTRACT BOOK
IMIBIC BUILDING
CONFERENCE ROOM
CÓRDOBA, 30-31 MAY, 2018

IIe. Characterization of human dry sweat metabolome by gas chromatography and liquid chromatography coupled to mass spectrometry and evaluation of sampling protocols

Authors: *María del Mar Delgado-Povedano[1,2,3,4], Laura de los Santos Castillo-Peinado[1,2,3,4], Mónica Calderón-Santiago[1,2,3,4], María Dolores Luque de Castro[1,2,3,4], Feliciano Priego-Capote[1,2,3,4]*
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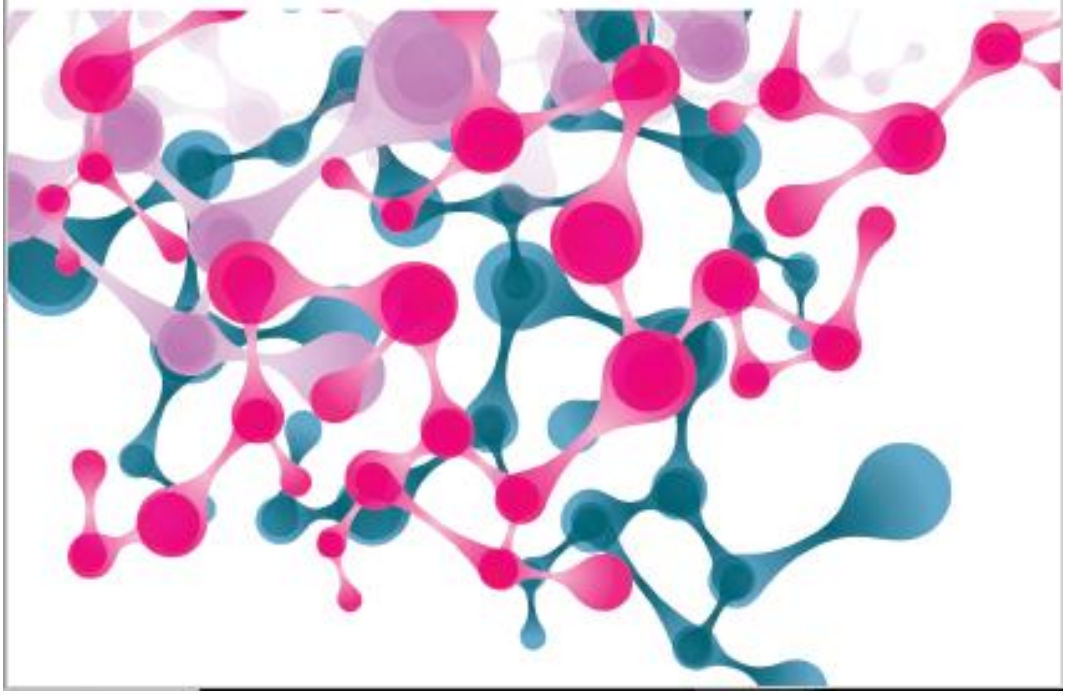
Fresh sweat has recently gained popularity as clinical sample in metabolomic analysis as this biofluid can be non-invasively sampled and its composition is modified by certain pathologies. In fact, human sweat has been recently used in metabolomic studies to discriminate between lung cancer patients and risk factor individuals. However, although sampling of dry sweat seems to be more stable than collection of fresh sweat because the former should be more reproducible, the whole composition of dry sweat is still unknown and there is a lack of strategies for its analysis, from collection to determination. With the final aim of characterizing the metabolome of dry sweat and comparing it with that of fresh sweat, two different sampling strategies for dry sweat (using a gauze or a paper, in both cases impregnated with different solvents) were evaluated by metabolomic analysis using GC–TOF/MS and LC–QTOF MS/MS. Three solvents were tested to check their influence on metabolites coverage. Among the tested strategies, paper with 50% (v/v) ethanol–phosphate buffer was the most suited option to obtain a representative snapshot of dry sweat metabolome. One hundred fifty-six compounds were tentatively identified by combination of the two platforms including interesting families of metabolites such as carnitines, sphingolipids and N-acyl-amino acids, among others. The comparison of the metabolomic profiles of fresh and dry sweat allows concluding that dry sweat is better for analysis of low polar metabolites while fresh sweat is more suited for polar compounds. As most of the identified metabolites are involved in key biochemical pathways, this study opens new possibilities to the use of dry sweat as source of metabolite biomarkers of specific disorders such as cancer. In fact, N-acetyl-amino acids such as acetylhistidine had been previously found at high-significantly lower concentration in urine from prostate cancer patients than in healthy individuals.

8th IMIBIC YOUNG INVESTIGATORS MEETING

IMIBIC Building • Conference Room • Córdoba, 30-31 May, 2017

Abstract Book



IVe. Metabolomics analysis of human sweat by gas chromatography-time of flight/mass spectrometry

Authors: *M.M. Delgado-Povedano[1,2,3], M. Calderón-Santiago[1,2,3], B. Jurado-Gámez[3,4], M.D. Luque de Castro[1,2,3], F. Priego-Capote[1,2,3]*

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Human sweat is a promising biofluid to be implemented in clinical studies by its composition and non-invasive sampling. In fact, sweat has been used in metabolomics studies to discriminate lung cancer patients versus risk factor individuals. Elucidation of sweat metabolome has so far been done using three analytical platforms (NMR, LC–MS and GC–MS). A total of 103 metabolites have been detected by combination of the three platforms, being GC–MS the approach providing the highest coverage, with detection of more than 60% of them. However, these studies did not pay attention to non-polar metabolites, presumably a minor fraction. The detection of non-polar compounds would increase the interest of sweat for clinical studies. With these premises, a method for metabolomics analysis of human sweat by gas chromatography–time of flight/mass spectrometry (GC–TOF/MS) in high resolution mode has been developed to expand the detection of compounds, with special emphasis on non-polar compounds. Different sample preparation strategies were compared to check their effect on the profile of sweat metabolites. The performance of three extractants with different polarity and a deproteinization protocol were compared by using a dual approach: (1) application of a derivatization protocol prior to GC–MS and, (2) direct injection. One hundred forty-three compounds were tentatively identified by high resolution MS. Mainly lipids, but also other interesting metabolites were identified. It is worthy to distinguishing the ability of the GC–MS protocol to detect lipids, family that constituted around 32% of the compounds detected. Among the tested protocols, methyoximation plus silylation after liquid–liquid extraction with dichloromethane was the most suited option to obtain a representative snapshot of sweat metabolome. As most of the identified metabolites are involved in key biochemical pathways, this study opens new possibilities to the use of sweat in searching for lung cancer biomarkers.

CREANDO REDES DOCTORALES

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SEDE UCO

Identificación tentativa de extractos enzimáticos acuosos de *Agaricus bisporus* con actividad antivirus de la hepatitis C mediante LC–MS/MScromatografía de líquidos acoplada a espectrometría de masas en tándem en modo de alta resolución

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Summary

Aqueous enzymatic extracts of edible mushroom *Agaricus bisporus* (AbAEE) have been studied as a key source of biological active compounds, among which those with antiviral activity against hepatitis-C virus are of special interest. Searching for a better knowledge of AbAEE, a new approach based on sequential fractionation with solvents of different polarity is here proposed to improve the tentative identification of AbAEE components by liquid chromatography–tandem mass spectrometry (LC–QTOF MS/MS) in high resolution mode. Fifty five out of one hundred twenty-three total metabolites were tentatively identified for the first time by combination of MS and MS/MS information. The newly identified compounds include amino acids, sugars, carboxylic, fatty and cinnamic acids, phospholipids, and purines as the most outstanding, thus demonstrating that fractionation based on sequential liquid–liquid extraction followed by LC–QTOF MS/MS is a suitable option to obtain a wide, representative snapshot of AbAEE composition.

Resumen

Se han estudiado extractos enzimáticos acuosos del hongo comestible *Agaricus bisporus* (AbAEE) como fuente clave de compuestos biológicos activos, entre los cuales son de especial interés los que tienen actividad antiviral contra el virus de la hepatitis C. Buscando un mejor conocimiento de la composición de AbAEE, se propone un nuevo enfoque basado en el fraccionamiento secuencial con disolventes de diferente polaridad para mejorar la identificación tentativa por cromatografía de líquidos–espectrometría de masas en tándem (LC–QTOF MS/MS) en modo de alta resolución. Cincuenta y cinco del total de ciento veintitrés metabolitos tentativamente identificados por combinación de datos de MS y MS/MS lo fueron por primera vez. Los nuevos compuestos identificados incluyen aminoácidos, azúcares, ácidos carboxílicos, grasos y cinámicos, fosfolípidos y purinas como los más destacados, demostrando de este modo que el fraccionamiento basado en la extracción líquido–líquido secuencial previa a LC–QTOF MS/MS es una opción adecuada para obtener una huella amplia y representativa de la composición de AbAEE.

7th IMIBIC YOUNG INVESTIGATORS MEETING

ABSTRACT BOOK

IMIBIC BUILDING
CONFERENCE ROOM
CÓRDOBA, 10-11 MAY, 2016

VI.c Recent advances in human sweat metabolomics for lung cancer screening.

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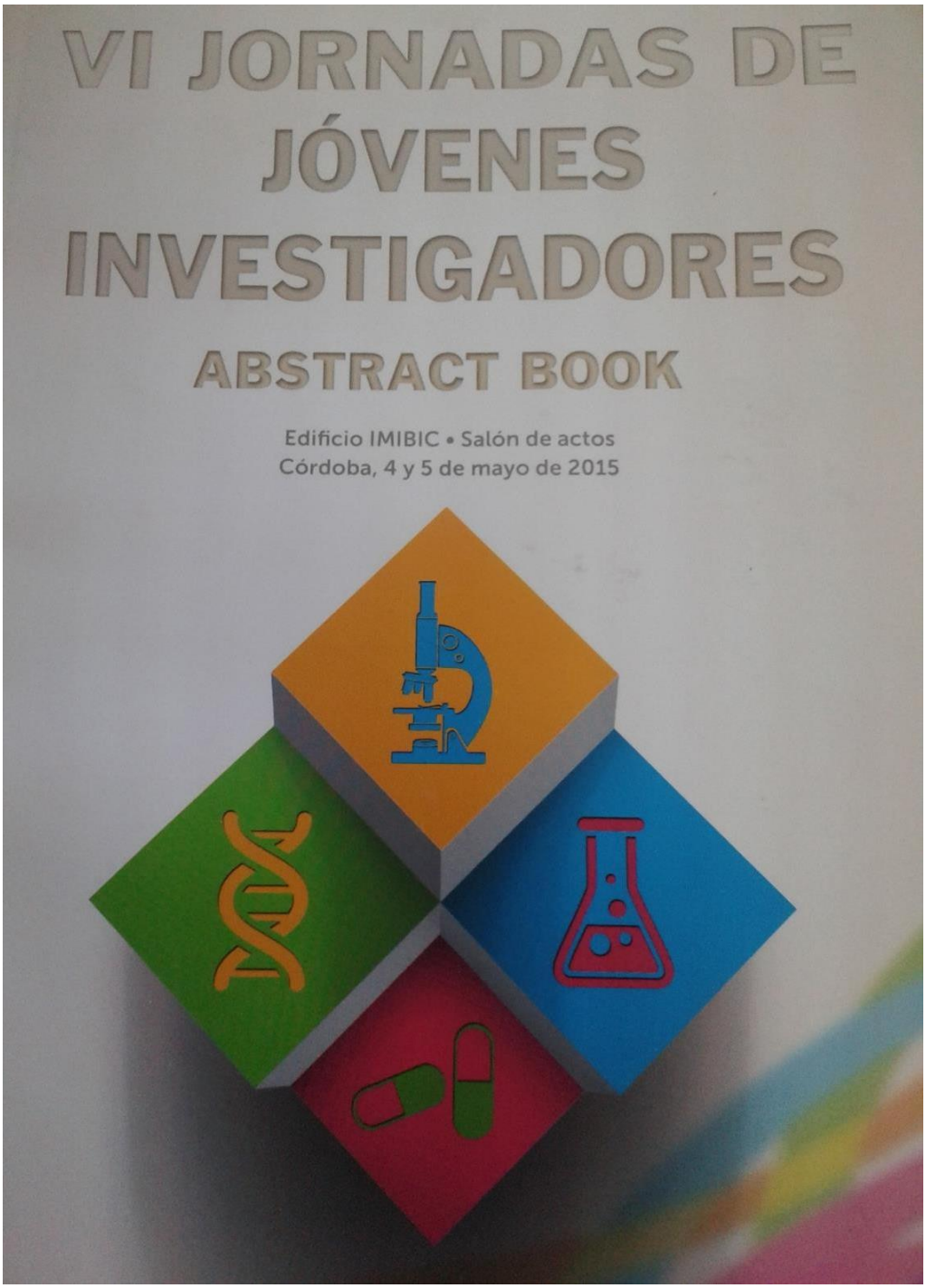
Group: GC21 Metabolomics Identification/Quantification of Bioactive Components.

Lung cancer is the leading cause of cancer related mortality owing to the advanced stage at which it is usually detected and the invasive nature of the existing diagnostic tests. For this reason, different biofluids have been studied to find new screening tools to detect lung cancer at earlier stages. However, these studies need to be validated to find a robust panel of markers. In this research, sweat samples from two batches (a first cohort of 35 patients recruited from March to August 2012 and a second cohort composed by 34 patients recruited from November 2012 to October 2014) were combined to increase the robustness of potential biomarkers. First of all, the variability between cohorts and the individual predictive capability of each metabolite were studied. The proposed biomarkers panels was oriented to discriminate between lung cancer patients and smokers as control individuals of the two cohorts, the samples from whom were collected and analyzed at different times. The resultant two panels of metabolites were configured using the PanelomiX tool as an attempt to reduce false negatives (at least 95% specificity) and false positives (at least 95% sensitivity). The first panel (96.9% specificity and 81.1% sensitivity), was composed by histidine, monoglyceride MG(22:2), nonanedioic acid, suberic acid, and a tetrahexose, while the second panel (84.4% specificity and 97.3% sensitivity) was composed by the monoglyceride MG(22:2), phenylalanine, a tetrahexose, a trisaccharide phosphate, and urocanic acid.


VI JORNADAS DE JÓVENES INVESTIGADORES
ABSTRACT BOOK
Edificio IMIBIC • Salón de actos
Córdoba, 4 y 5 de mayo de 2015

21. Advances in the search for biomarkers to develop a screening tool for the diagnosis of lung cancer

Authors: *M.M. Delgado-Povedano, M. Calderón-Santiago, B. Jurado-Gámez, F. Priego-Capote, M.D. Luque de Castro*

Group: GC21 *Metabolomics. Identification of bioactive components*

Lung cancer is the carcinogenic disease with the highest mortality rate owing to the advanced stage at which it is usually diagnosed. For this reason, different biofluids have been studied to find new screening tools to detect lung cancer at earlier stages. In this research, a non-invasive biofluid as human sweat was used as clinical sample to develop a screening tool for lung cancer. With this aim, a method based on analysis of the metabolites in sweat to discriminate between patients with lung cancer versus smokers as control individuals was developed. In a first step, a cohort of 23 patients diagnosed with lung cancer and 18 control individuals was studied. The capability of the metabolites identified in sweat to discriminate between both groups of individuals was studied and, among them, a trisaccharide phosphate presented the best independent performance in terms of the specificity/sensitivity pair (80 and 72.7%, respectively).

Additionally, two panels of metabolites were configured using the PanelomiX tool. The first panel (80% specificity and 69% sensitivity) was composed by suberic acid, a tetrahexose and a trihexose, while the second panel (69% specificity and 80% sensitivity) included nonanedioic acid, a trihexose and the monoglyceride MG(22:2). Thus, the combination of the five metabolites led to a single panel providing 80% specificity and 79% sensitivity. The study was expanded with a second cohort including 34 lung cancer patients and 14 control individuals. The new panel providing 85% specificity and 87% sensitivity was composed by urocanic acid, a tetrahexose, two dipeptides (GluVal and γ-GluLeu), and a monoglyceride MG(22:2). Finally, a first approach to find metabolomics differences in sweat to discriminate between early and advanced stages of lung cancer is being carried out.

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III CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN EN AGROALIMENTACIÓN

CREANDO REDES

CÓRDOBA, 18 Y 19 DE NOVIEMBRE DE 2014
EN EL RECTORADO DE LA UNIVERSIDAD DE CÓRDOBA
AVDA. MEDINA AZAHARA 5

Análisis cuantitativo del perfil de aminoácidos en sudor humano mediante cromatografía líquida–espectrometría de masas de triple cuadrupolo

Introducción

El sudor constituye una muestra escasamente utilizada en análisis clínico, a pesar de la facilidad de su obtención y el carácter no invasivo de su muestreo. Entre los usos destacables de este fluido biológico se encuentra su aplicación a la diagnosis de la fibrosis quística [1], el control de la relación hidro-electrolítica durante el ejercicio humano [2,3] y la detección de la presencia de sustancias tóxicas en el organismo [4,5]. La metabolómica, la más reciente de las grandes ómicas, está adquiriendo un gran desarrollo en su vertiente clínica, especialmente en la búsqueda de biomarcadores de enfermedades, por lo que constituye una disciplina prometedora en el uso del sudor para esta búsqueda.

En un estudio reciente desarrollado en nuestro Grupo de Investigación, se utilizó el sudor como muestra para la discriminación metabolómica entre sujetos con y sin cáncer de pulmón (CP) mediante un perfil global obtenido con un equipo de cromatografía líquida–tiempo de vuelo/espectrometría de masas (LC–TOF/MS). El tratamiento quimiométrico de los datos obtenidos permitió obtener un panel de biomarcadores de CP compuesto por tres metabolitos y caracterizado por 100% de especificidad y el 81% de sensibilidad [6–8]. Estos resultados hicieron deseable el desarrollo de la siguiente investigación, que ha constituido el trabajo fin de máster de la autora y el comienzo de su Tesis Doctoral.

Objetivos

El **objetivo genérico** de la investigación fue desarrollar una plataforma analítica robusta y validada para obtener perfiles cuantitativos de metabolitos en sudor.

Los **objetivos concretos** fueron:

Objetivo 1: Optimizar un método muy sensible y selectivo basado en LC–MS/MS en modo de monitorización de reacciones seleccionadas (SRM) para el análisis confirmatorio y cuantitativo de aminoácidos en sudor humano.

Objetivo 2: Proponer diferentes formas de preparación de la muestra de sudor (dilución, extracción en fase sólida automatizada, microextracción en fase sólida) para minimizar o evitar el efecto matriz del sudor en la determinación de los metabolitos problema.

Objetivo 3. Caracterizar el método total (resultado de combinar la etapa de preparación de la muestra con la de detección) evaluando sus propiedades analíticas. Esta caracterización soporta el uso del sudor como muestra analítica para obtener un perfil de aminoácidos teniendo en cuenta el número de patologías en las que está implicado el metabolismo de estos compuestos.

Materiales y métodos

El perfil de analitos estuvo compuesto por todos los aminoácidos proteinogénicos además de otros tales como carnitina, citrulina, taurina, y creatinina, que fueron seleccionados por su importancia biológica.

Para el análisis de estos aminoácidos se utilizó la cromatografía líquida (LC) con detección mediante espectrometría de masas (MS/MS) usando un espectrómetro de triple cuadrupolo (QqQ) Agilent 6460 equipado con una fuente de ionización por electrospray (ESI) Jetstream®. Los datos se procesaron mediante un software Workstation MassHunter para el análisis cualitativo y cuantitativo.

Las muestras de sudor para el desarrollo y caracterización del método se tomaron de voluntarias sanas en el Hospital Reina Sofía, provocando la sudoración mediante un inductor de sudor Webster. El sudor se recogió en un colector Macroduct.

Para la preparación de la muestra se compararon dos protocolos: Uno de dilución de la muestra y otro de extracción en fase sólida (SPE). En el primero, se preparó un pool de sudor con todas las muestras y se tomaron alícuotas, que se utilizaron tal cual o enriquecidas a dos concentraciones (50 y 250 ng/mL) con un multipatrón de los aminoácidos en medio acuoso 0.1 M en ácido fórmico (AF). En el segundo, las alícuotas se diluyeron con HCl 0.1M y se sometieron a microSPE (en formato centrífugo).

Para la separación cromatográfica se utilizó una columna analítica C18 en fase reversa termostatizada a 25 °C. Las fases móviles fueron agua y acetonitrilo–agua 90:10, ambos con formiato amónico 5 mM como agente ionizante. La bomba del LC se programó a 0.5 mL/min en modo gradiente.

La detección se llevó a cabo en ionización positiva en modo SRM con una resolución *m/z* de 1. Para la cuantificación de los aminoácidos en el sudor se utilizó el área del pico obtenido para la transición de cada analito, que coincidió con el tiempo de retención cromatográfico proporcionado por la disolución multipatrón. Los modelos de calibración para cada analito se desarrollaron usando disoluciones multipatrón a diferentes concentraciones mediante la dilución de la disolución madre con 0.1 M FA en agua.

Principales aportaciones

Se optimizó la preparación de la muestra (tanto la dilución necesaria para evitar el efecto matriz como el tipo de cartucho para la SPE), la separación cromatográfica (la columna, el tipo de gradiente y el pH), la ionización (parámetros de la fuente), el método SRM (el voltaje del Q1 y la energía de colisión) y la transición para la cuantificación de los aminoácidos.

Se evaluó el potencial del sudor para la obtención del perfil cuantitativo de aminoácidos mediante un estudio exhaustivo de la preparación de la muestra, en el que se demostró que el SPE en el formato centrífugo era la opción que proporcionaba unas mejores características analíticas del método global.

El pequeño volumen de sudor muestreado en cada individuo hace imprescindible el uso de microformatos para la preparación de la muestra, como es el caso de las microcolumnas de tipo centrífugo utilizadas en esta investigación.

La dilución del sudor es necesaria para disminuir el efecto matriz y conseguir así la cuantificación absoluta de los aminoácidos.

Los modelos de calibración obtenidos son buenos, con coeficientes de regresión superiores a 0.98 para todos los analitos, excepto para la cisteína, que fue del 0.97. Los límites de detección y de cuantificación obtenidos están entre los pg/mL y los ng/mL.

Los aminoácidos se comportan de forma diferente entre sí; por tanto, se han de adoptar soluciones de compromiso cuando se realiza su determinación en un solo análisis.

El sudor puede ser una muestra excelente para la obtención del perfil cuantitativo de aminoácidos en estudios clínicos; lo que es de interés si se tiene en cuenta el papel que tienen estos compuestos como biomarcadores.

Investigación prevista

-Estudio en sudor de los perfiles de otras familias de metabolitos potenciales biomarcadores.

-Validación de los métodos mediante aplicación a muestras de sudor de pacientes de CP, de otras patologías y a individuos sanos. Aplicación a una población amplia en diferentes grados de desarrollo de CP.

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Anexo IX. Comunicaciones orales en congresos de medicina

Annex IX. Oral communications in medicine meetings

1. **Cáncer epidermoide pulmonar: Identificación de metabolitos en el sudor según estadios iniciales o avanzados**

   F. Montoro-Ballesteros, M.M. Delgado-Povedano, C. Esteban-Amarilla, A. Palomares-Muriana, M. Calderón-Santiago, F. Priego-Capote, M.D. Luque de Castro, B. Jurado-Gámez

   49º CONGRESO NACIONAL DE LA SOCIEDAD ESPAÑOLA DE NEUMOLOGÍA Y CIRUGÍA TORÁCICA (SEPAR)

   Granada, 2016

   Tipo de evento: Póster en Congreso **Ámbito:** Nacional

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2. **Metabolómica aplicada al sudor de pacientes con cáncer epidermoide en estadio inicial**

   F. Montoro-Ballesteros, M.M. Delgado-Povedano, C. Esteban-Amarilla, A. Palomares-Muriana, M. Calderón-Santiago, F. Priego-Capote, M.D. Luque de Castro, B. Jurado-Gámez

   42º CONGRESO NEUMOSUR

   Huelva, 2016

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Regional

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3. **Human sweat metabolomics for lung cancer prediction**

   M. Calderón-Santiago, M.M. Delgado-Povedano, F. Priego-Capote, B. Jurado-Gámez, M.D. Luque de Castro

   IABR SUMMIT 2015 - IABR's 10th ANNIVERSARY CONFERENCE

   Viena, 2015

   Tipo de evento: Comunicación oral en Congreso **Ámbito:** Internacional

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GRA
NADA
49 CONGRESO NACIONAL SEPAR
10-13 de junio
2016

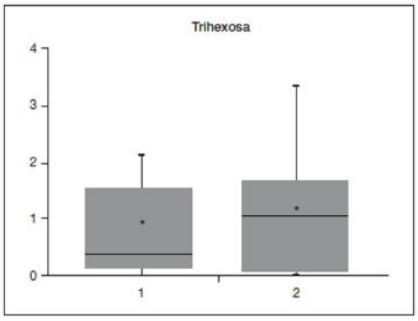

Sociedad Española de Neumología y Cirugía Torácica SEPAR
ALAT
ISSN: 0300-2896
Órgano Oficial de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR), la Asociación Latinoamericana de Tórax (ALAT) y la Asociación Iberoamericana de Cirugía Torácica (AIACT)
ARCHIVOS DE
Bronconeumología
VOLUMEN 52 | ESPECIAL CONGRESO 1 | JUNIO 2016
49 Congreso Nacional de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR)
Granada, 10-13 de junio de 2016
www.archbronconeumol.org
ELSEVIER

CÁNCER EPIDERMOIDE PULMONAR: IDENTIFICACIÓN DE METABOLITOS EN EL SUDOR SEGÚN ESTADIOS INICIALES O AVANZADOS

F. Montoro Ballesteros, M.M. Delgado Povedano, C. Esteban Amarilla., A. Palomares Muriana, M. Calderón, F. Prieto Capote, M.D. Luque de Castro y B. Jurado Gámez

Hospital Universitario Reina Sofía.

Introducción: Evaluar si la metabolómica aplicada a una muestra de sudor presenta diferencias en los compuestos de cáncer epidermoide de pulmón estadio inicial (CEP I-II) respecto a estadios más avanzados (CEP III-IV).
Material y métodos: Se incluyeron 19 muestras de sudor pertenecientes en 9 casos a pacientes con CEP I-II y en 10 con CEP III-IV. La recolección del sudor se llevó a cabo mediante técnica estandarizada y para el análisis metabolómico se utilizó un cromatógrafo de líquidos acoplado a un espectrómetro de masas de alta resolución (LC-QTOF) con ionización por electrospray. En cada grupo se estudió el cambio de la concentración relativa de cada uno de los 16 compuestos que fueron previamente identificados en sudor con diferencias significativas entre sujetos sin CP respecto a pacientes. Los datos se procesaron con el software MassHunter Workstation y se realizó Análisis de Cambio para detectar las diferencias de concentración relativa de metabolitos entre los grupos.
Resultados: Los pacientes tuvieron una edad de 64 años (61-71), 16 de los cuales fueron hombres (854%) con un IMC de 26. Para encontrar diferencias entre los metabolitos de los pacientes con CEP I-II y CEP III-IV, se llevó a cabo un análisis de cambio. Una trihexosa fue el único compuesto con cambio de concentración relevante entre sujetos con CP I-II y sujetos con CP III-IV (FC: −2,173), siendo su presencia mayor en el grupo con CP en estadio avanzado. La figura resume los compuestos con mayores diferencias en concentración relativa entre sujetos con CP en estadios precoces y estadios avanzados. En 6 de los 9 sujetos (67%) en estadios iniciales estaban asintomáticos y fue un hallazgo casual radiológico.



Conclusiones: i) el presente estudio aporta conocimiento sobre la importancia del metaboloma del sudor para discriminar entre una población con riesgo de padecer CP y pacientes con CEP en diferentes estadios; ii) Los resultados muestran que en el análisis de cambio el único compuesto que mostró diferencias de concentración relativa entre sujetos con CP en estadios iniciales frente a avanzados fue una trihexosa. iii) La ausencia de síntomas en sujetos con CEP I-II es frecuente y la trihexosa en sudor puede indicar la necesidad de realizar una TAC de baja radicación para ampliar el estudio.

CARACTERÍSTICAS DIFERENCIALES DEL CÁNCER DE PULMÓN EN PACIENTES CON EPOC

E. Marijuán, O. Llaguno, I. Murga, A. Gómez-Larrauri, C. Sánchez, J.L. Lobo, P. Sobradillo y D. Bravo

Hospital Universitario de Araba.

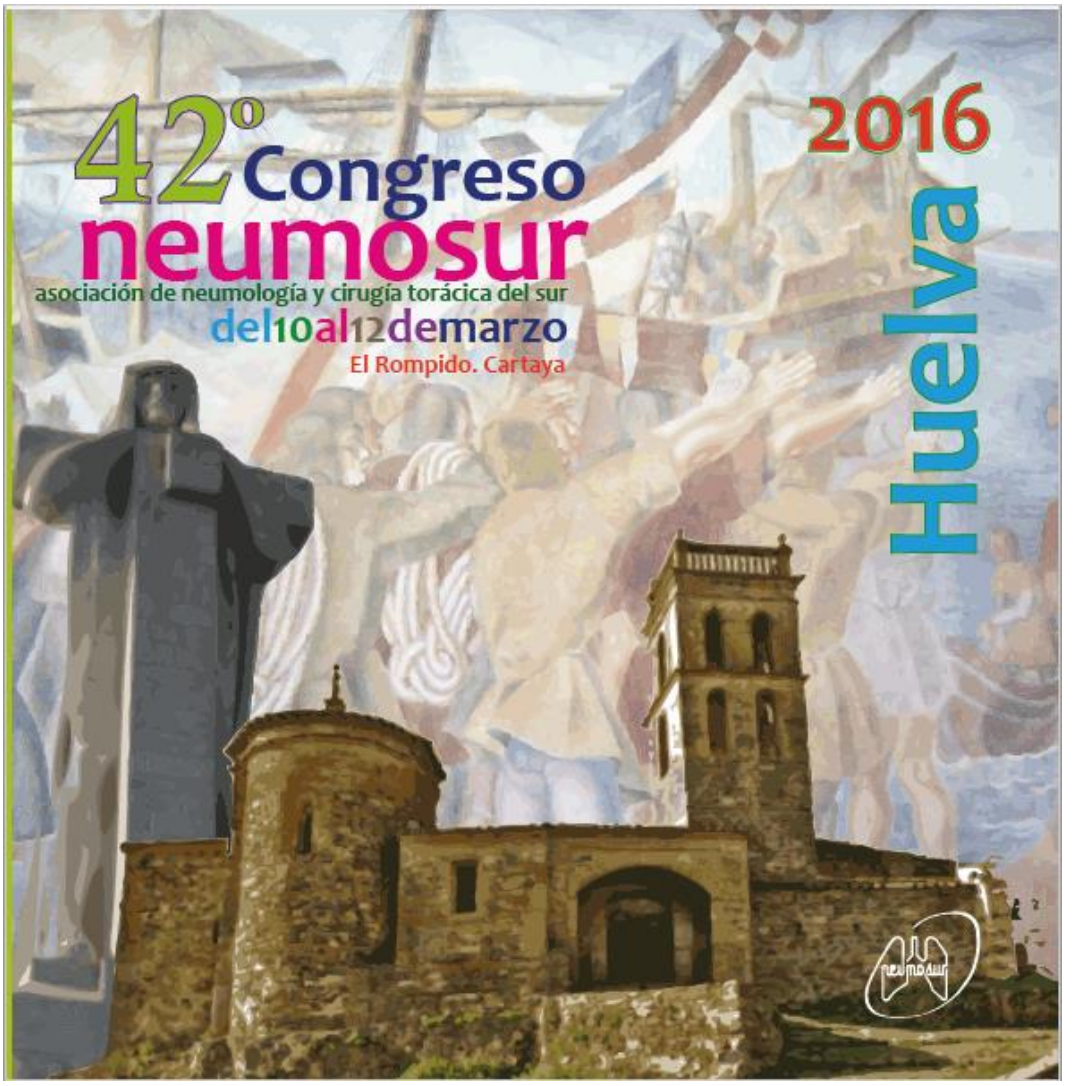
Introducción: El cáncer de pulmón y la EPOC comparten un mismo factor de riesgo, el tabaquismo, siendo, además, la EPOC un factor de riesgo independiente para desarrollar cáncer de pulmón. El objetivo del estudio fue analizar las características epidemiológicas y de presentación así como la supervivencia de los pacientes con neoplasia pulmonar primaria y EPOC, y compararlo con los pacientes sin EPOC.
Material y métodos: Se realizó un estudio retrospectivo y descriptivo en el que se recogieron los pacientes con diagnóstico de neoplasia pulmonar primaria, incluidos en el Comité de Tumores Pulmonares desde septiembre de 2011 hasta diciembre de 2013. Se recogieron distintas variables epidemiológicas, clínicas y funcionales así como los datos de supervivencia. Las variables se describen como porcentajes, media y desviación estándar y para las comparaciones entre grupos se realizan las pruebas de chi-cuadrado y de Mann-Whitney, según las variables a comparar. Se realizó el estudio de supervivencia mediante las gráficas Kaplan-Meier. Se consideró estadísticamente significativa una $p < 0,05$.
Resultados: Se incluyeron 201 pacientes, de los cuales 63 (31,3%) tenían diagnóstico concomitante de EPOC. De estos, 53 eran hombres (84,1%). La edad media de los pacientes al diagnóstico fue de 64,57 ± 10,31, sin diferencias significativas entre los dos grupos. Los pacientes con EPOC eran más frecuentemente exfumadores y presentaban un mayor IA tabáquico. Se dividió a los pacientes en función del grado de obstrucción (GOLD 1-4) sin observarse diferencias en la distribución en cuanto a estadiaje y estirpe histológica. Se observó menor supervivencia en los pacientes con diagnóstico concomitante de EPOC.
Conclusiones: La prevalencia de pacientes con diagnóstico concomitante de EPOC y cáncer es del 31,3%. La estirpe histológica más frecuente en los pacientes con EPOC fue el epidermoide y en los no EPOC el adenocarcinoma. No se observan diferencias significativas en el estadiaje ni en la histología en función de la gravedad de la obstrucción al flujo aéreo. Los pacientes con EPOC presentan menor supervivencia que los sin EPOC.

CARCINOMA BRONCOGÉNICO Y ALGUNOS FACTORES ASOCIADOS

F. Oliveri Aruete, A. Candelario Cáceres, A.M. Gómez Arenas y M.A. Fernández Jorge

Hospital Río Carrión.

Introducción: Analizar las características del cáncer de pulmón, su asociación con el tabaquismo; así como la estirpe histológica, síntomas, estadio y tratamiento más frecuente.
Material y métodos: Estudio retrospectivo, descriptivo y observacional donde se incluyen los pacientes diagnosticados de cáncer pulmonar desde enero-2014 hasta octubre-2015. Los datos han sido recogidos mediante el sistema de codificación de nuestro hospital y la revisión de las historias clínicas. Para el estudio estadístico hemos utilizado el SPSS versión 20.0.
Resultados: Total de 135 pacientes, 83,7% hombres. Edad media de 72 años (DE ± 11) mínima de 34 y máxima de 105. Fumadores activos al momento del diagnóstico 44,4%, exfumadores 48,1%, nunca fumadores 7,4% (de los cuales, 7 mujeres y 3 hombres). El síntoma inicial más frecuentes fue la tos 23%, seguido de la hemoptisis 15,6% y la disnea 11,9%. Metástasis al diagnóstico 50,2%: múltiples 21,5%, digestivas 8,9% y pulmón contralateral en 8,9%. Estadios más frecuentes al momento del diagnóstico: 38,5% IV, 12,6% IIIB, 12,6% IIIA. Pacientes con antecedentes


42º Congreso
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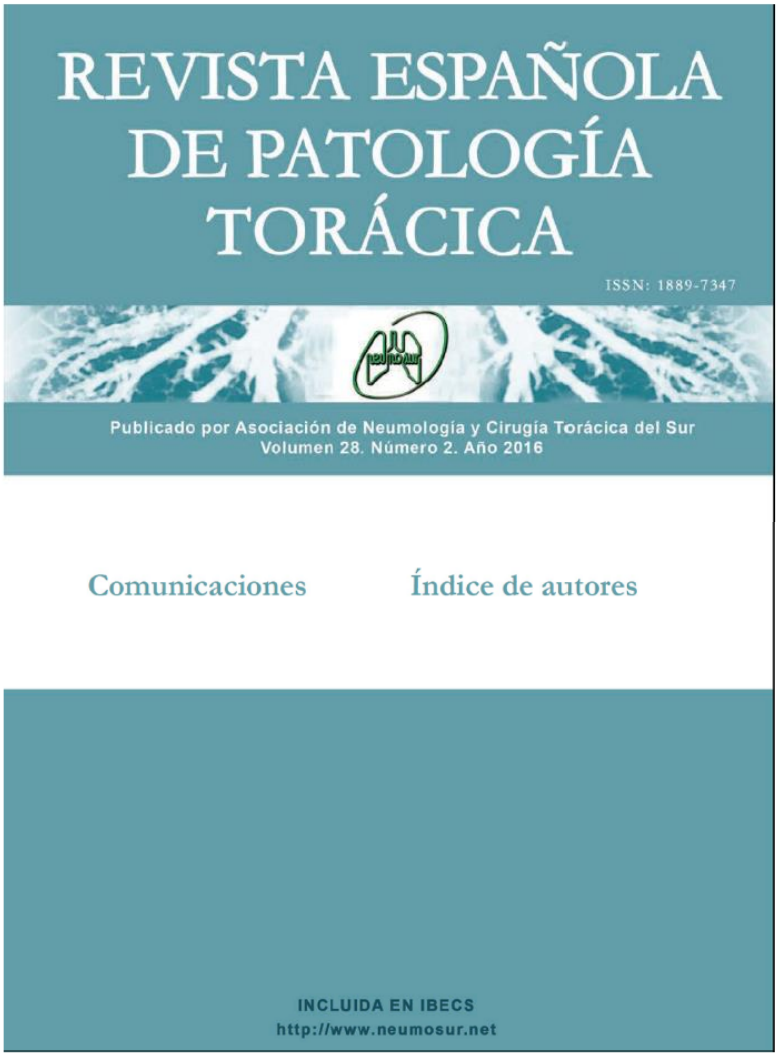
asociación de neumología y cirugía torácica del sur
del 10 al 12 de marzo
El Rompido. Cartaya
2016
Huelva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una edad media 70 ± 9 años y un FEV1 medio del 50,7 ± 17,8%. En 458 pacientes (49,5%) se establecía un diagnostico de EPOC según fenotipo de GesEPOC. Entre estos pacientes, las opciones de tratamiento más frecuentes para el fenotipo no agudizador fueron LAMA/LABA (42%), LAMA/LABA/CI (26%), LAMA (9,3%), LABA (4,4%), para el fenotipo ACOS (solapamiento EPOC - Asma) LAMA/LABA/CI (61%), LAMA/LABA (18,8%), LABA/CI (17,6%), LAMA (1,5%), para el fenotipo agudizador con enfisema LAMA/LABA/CI (65,3%), LAMA/LABA (8,4%), LABA/CI (8,4%), LAMA (2,8%) y para el fenotipo agudizador con bronquitis crónica LAMA/LABA/CI (70,4%), LAMA/LABA (8,9%) y LABA/CI (7,1%).

Conclusiones: según los datos de EPOCONSUL de los hospitales del ámbito Neumosur, existen prescripciones de tratamiento correctas, principalmente para los pacientes con exacerbaciones frecuentes, siendo el fenotipo no agudizador donde se aprecia menor adecuación a GesEPOC.

METABOLÓMICA APLICADA AL SUDOR DE PACIENTES CON CÁNCER EPIDERMOIDE EN ESTADÍO INICIAL

F. Montoro Ballesteros [1], M.M. Delgado Povedano [2], C. Esteban Amarilla [1], A. Palomares Muriana [1], M. Calderón Santiago [2], F. Prieto Capote [2], M. Luque de Castro [2], B. Jurado Gámez [1].

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Introducción: el cáncer de pulmón (CP) es la principal causa de muerte tumoral en los países desarrollados. Las nuevas omicas hacen posible la aplicación de métodos no invasivos para el cribado de enfermedades y el sudor es una muestra cuya potencialidad en el campo tumoral es importante y novedosa.

Metodología: objetivo: Evaluar si la metabolómica aplicada a una muestra de sudor presenta diferencias en los compuestos de cáncer epidermoide de pulmón en estadio inicial (CEP I-II) respecto a un grupo control. Pacientes y métodos: se incluyeron 19 muestras de sudor pertenecientes a 9 pacientes con CEP I-II y 10 sujetos control (tabaquismo >25 paquetes/año). El sudor se recogió según técnica estandarizada y fué inmeditamente congelado a -80°C hasta el análisis metabolómico, para lo que se utilizó un cromatógrafo de líquidos, acoplado a un espectrómetro de masas de alta resolución (LC-QTOF) con ionización por electrospray. Se realizó un análisis de cambio para detectar las diferencias de concentración relativa de metabolitos entre grupos.

Resultados: no se observaron diferencias en las características basales de los sujetos incluidos en los dos grupos. En la presentación clínica destaca que más de la mitad de los enfermos con CEP I-II (67%) no tuvieron sintomatología atribuible al tumor. En el análisis de cambio, el compuesto con mayor diferencia entre sujetos sin CP respecto a pacientes con CP I-II en concentración relativa fue una tetrahexosa, que mostró un AC = ?4,020, igual comportamiento se observó en un trisacárido fosfato (AC = -1,735) y, en menor medida, en un lípido sulfónico (AC = ?1,918).

Conclusiones: En más de la mitad de los casos de CEP I-II, los pacientes no acuden a consulta por síntomas. En el análisis de cambio existen metabolitos con potencialidad para discriminar entre sujetos fumadores con riesgo de padecer CP y aquellos con CEP I-II. Este hallazgo es relevante y puede tener aplicabilidad clínica en el cribado del cáncer de pulmón.

INFLUENCIA DE LAS COMORBILIDADES EN LA MORTALIDAD DE PACIENTES CON EPOC ESTABLES

A. Arnedillo Muñoz [1], J.L. López-Campos Bodineau [2], F. Casas Maldonado [3], P. Cordero Montero [4], I. Alfageme Michavilla [5].

[1] UGC de Neumología y Alergia. Hospital U. Puerta del Mar. Cádiz. [2] Unidad Médico-Quirúrgica de Enfermedades Respiratorias. Hospital U. Virgen del Rocío. Sevilla. [3] Servicio de Neumología. Hospital U. San Cecilio. Granada. [4] Servicio de Neumología. Hospital Infanta Cristina. Badajoz. [5] Servicio de Neumología. Hospital U. Valme. Sevilla.

Introducción: existen estudios que han demostrado una mayor mortalidad en los pacientes con EPOC asociado a las comorbilidades y en algunos de ellos asociados a determinadas comorbilidades, aunque los resultados muestran mucha variabilidad, dependiendo de la población estudiada. Nuestro objetivo fue estudiar la relación entre las comorbilidades y la mortalidad por cualquier causa en pacientes con EPOC en fase estable, así como la influencia de cada una de ellas en dicha mortalidad en nuestro ámbito geográfico.

Metodología: estudio observacional prospectivo longitudinal multicéntrico de una cohorte de pacien-

AGENDA VERSION 2 AS OF SEPTEMBER 10

IABR summit 2015 - IABR's 10th anniversary conference

hosted by Marco Freek

conference venue: Schonbrunn Palace, Apotheker Wing, Meidlinger Portal, Grunbergstreet, Vienna

NO AUDIO RECORDING, VIDEO RECORDING OR PHOTOGRAPHY IS ALLOWED WITHOUT THE HOST'S PERMISSION.

Monday, September 14, 2015

		ROOM MARIA THERESIA	ROOM FRANZ JOSEPH
	13:00 - 13:30	*Keynote: New perspectives in clincial breath biomarker research – From voodoo to vision (Wolfram Miekisch)*	*Keynote: Progress toward a breath test for bacterial respiratory infections (Jane Hill)*
	13:35 - 13:50	*Lack of vision and investments for development of diagnostic breath tests: Towards personalizing medicine (Anil Modak)*	*BiOAIR - An interdisciplinary project for lung cancer biomarker identification in exhaled breath (Kristin Schallschmidt)*
	13:50 - 14:05	*Linking the gut microbiome and Volatile Organic Compounds in breath: a case study on Crohn's Disease (Agnieszka Smolinska)*	*Human sweat metabolomics for lung cancer prediction (Monica Calderon-Santiago)*
	14:05 - 14:20	*Breath biomarker studies using laser spectroscopy (Florian Schmidt)*	*Investigation of the effect of curative oesophago-gastric cancer resection on volatile organic compounds in exhaled breath using SIFT-MS (Sophie Doran)*
	14:20 - 14:35	*Malodor and Gingivitis: Clinical Evidence with Stannous Fluoride and Novel Oral Hygiene (Robert Gerlach)*	*Profiling volatile organic compounds in exhaled breath by TD-GC-TOF MS (Laura McGregor)*
	14:35 - 15:00	discussion	discussion
15:00 - 15:30		*Coffee Break*	
15:30 - 17:30		**C: EBC**	**D: medical monitoring**
		chairs: Joachim Pleil and Feliciano Priego-Capote	*chairs: Jörg-Ingo Baumbach and Haiyang Li*
	15:30 - 16:00	*Keynote: Human exhaled breath condensate (EBC) media: implementation of automated Quanterix SIMOA immunochemistry instrumentation (Joachim Pleil)*	*Keynote: Metabolic MCC/IMS-Profiles of Human Breath for Bedside Applications (Joerg-Ingo Baumbach)*
	16:05 - 16:20	*Variability of exhaled breath condensate (EBC) volume and pH using a feedback regulated breathing pattern (Michael Madden)*	*Real-time Monitoring Biomarkers in Breath Air by High-resolution Photoionization Ion Mobility Spectrometry and Mass Spectrometry (Haiyang Li)*
	16:20 - 16:35	*Exhaled Breath Condensate Biomarkers in Critically Ill, Mechanically Ventilated Adults (Michael Davis)*	*Online Measurement of FENO Profile by Fast High-resolution Ion Mobility Spectrometry (Liying Peng)*

Human sweat metabolomics for lung cancer prediction

M. Calderón-Santiago[a,b], M.M. Delgado-Povedano[a,b], F. Priego-Capote[a,b], B. Jurado-Gámez[b,c], M.D. Luque de Castro[a,b]

[a] Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071 Córdoba, Spain

[b] Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071 Córdoba, Spain

[c] Department of Respiratory Medicine, Reina Sofía University Hospital, Córdoba, Spain

Lung cancer is the carcinogenic disease with the highest mortality rate owing to the advanced stage at which it is usually detected; therefore, biomarkers for lung cancer detection at early stages are needed. On the other hand, sweat has recently gained popularity as a potential tool for diagnostics biomarker monitoring.

A two-step research has been developed:

First, an analytical method for analysis of human sweat by liquid chromatography mass spectrometry (LC–QTOF MS/MS) in high resolution mode was developed. Forty one compounds were identified by the MS/MS information obtained with a mass tolerance window below 4 ppm. Amino acids, dicarboxylic acids and other interesting metabolites such as inosine, choline, uric acid and tyramine were identified.

The second step was to use the developed method for analysis of sweat to discriminate between patients with lung cancer versus control individuals (smokers with an average of 37 pack-years*). The capability of the metabolites identified in sweat to discriminate between both groups was studied. In a first approach, a cohort of 23 patients diagnosed with lung cancer and 18 control individuals was studied. Two panels of three metabolites were configured using the PanelomiX tool, the first (80% specificity and 69% sensitivity) was composed by suberic acid, a tetrahexose and a trihexose, while the second panel (69% specificity and 80% sensitivity) included nonanedioic acid, a trihexose and the monoglyceride MG(22:2). Thus, the combination of the five metabolites led to a single panel providing 80% specificity and 79% sensitivity. Lately, this panel was improved expanding the cohort to 57 lung cancer patients and 32 control individuals. The new panel provided 85% specificity and 87% sensitivity, and it was composed by urocanic acid, a tetrahexose, the monoglyceride MG(22:2) and two dipeptides (GluVal and γ-GluLeu).

*Pack-years=(average cigarettes smoked per day/20) × (years of smoking).

Acknowledgements: the Spanish Foundation Respira (78/2013), the Spanish Ministerio de Economía y Competitividad (MINECO) and the FEDER Program (project no. CTQ2012-37428), and the Junta de Andalucía through an Excellence project (FQM-1602) are thanked for economical support.

Abreviaturas

Abbreviations

Abbreviations

A. bisporus, *Agaricus bisporus*; *A. niger*, *Aspergillus niger*; AbAEE, *Agaricus bisporus* aqueous enzymatic extracts; AbRE, *Agaricus bisporus* raw extracts; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ACE, angiotensin I-converting enzyme; ACN, acetonitrile; AOA, total antioxidant activity; APCI, atmospheric pressure chemical ionization; API, atmospheric pressure ionization; APPI, atmospheric pressure photoionization; AUC, area under the curve; BBD, Box-Behnken design; BD, biodiesel; BPC, base peak chromatogram; BSTFA, bis-(trimethylsilyl)-fluoroacetamide; CAPE, caffeic acid phenethyl ester; CBC, cannabichromene; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBDVA, cannabidivarinic acid; CBE, cannabielsoin; CBG, cannabigerol; CBGA, cannabigerolic acid; CBGV, cannabigerovarin; CBL, cannabicyclol; CBN, cannabinol; CD, circular dichroism; CE, capillary electrophoresis; CI, chemical ionization; CIL, chemical isotope labeling; c-SPμE, centrifugal solid-phase microextraction; DAD, diode array detector; DAG, diacylglycerol; Dansyl-Cl,1-dimethylaminonaphthalene-5-sulphonyl chloride; DART, direct analysis in real time; DE, degree of esterification; DESI, desorption electrospray ionization; DFI, direct fluid injection; DIMS, direct infusion mass spectrometry; DLLME, dispersive liquid–liquid microextraction; DMB, 1,2-diamino-4,5-methylene dioxybenzene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DSS, sodium trimethylsilylpropanesulfonate; DW, dried weight; DWGP, defatted wheat germ protein; EAE, enzyme assisted extraction method; EDP, 2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate; EFA, esterified fatty acid; EI, electron impact; ES, external standard; ESI, electrospray ionization; FA, formic acid; FAAS, flame atomic absorption spectrometry; FAB, fast atom bombardment; FAME, fatty acid methyl ester; F–C, Folin–Ciocalteu; FDA, Food and Drug Administration; FDNB, 1-fluoro-2,4-dinitrobenzene; FID, flame ionization detection; FRAP, ferrous reducing antioxidant power; FT–IR, Fourier transform infrared analysis; FWHM, full width at half maximum; GABA, γ-aminobutyric acid; GC, gas chromatography; GC–FID, gas chromatography–flame ionization detector; GC–FTIR, gas chromatography–Fourier transform infrared spectroscopy; GC–MS, gas chromatography–mass spectrometry; GC–MS/MS, gas chromatography–

tandem mass spectrometry; GC–TOF/MS, gas chromatography–time-of-flight/ mass spectrometry; GHz, gigahertz; GlyC, glycerol carbonate; HCV, hepatitis-C virus; HHP, high hydrostatic pressure; HMDB, Human Metabolome Database; ^{1}H-NMR, proton nuclear magnetic resonance; HPLC, high performance liquid chromatography; HPLC–DAD, high pressure liquid chromatography–diode array detection; HS, head-space; ICAT, isotope coded affinity tags; ICP, inductively coupled plasma; ICP-OES, inductively coupled plasma optical emission spectrometry; IL, ionic liquid; IT, ion trap; k_A, apparent breakdown rate constant; kHz, kilohertz; K_M, Michaelis-Menten constant; LC, liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LC–QTOF MS/MS, liquid chromatography quadrupole time-of-flight tandem mass spectrometry; LC–UV, liquid chromatograph coupled to a UV detector; LIF, laser induced fluorescence; LLE, liquid–liquid extraction; LLME, liquid–liquid microextraction; LOD, limit of detection; LOQ, limit of quantitation; LTQ, linear trap quadrupole; MAD, microwave-assisted digestion; MAE, microwave-assisted extraction; MALDI, matrix-assisted laser desorption/ionization; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MF, molecular feature; MG, monoglyceride; MHz, megahertz; MMCD, Madison Metabolomics Consortium Database; MPP, Mass Profiler Professional; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MTS, nano-thermosonication; MW, microwave(s); NEFA, no esterified fatty acid; NIST, National Institute of Standards and Technology; NMR, nuclear magnetic resonance; OPA, *o*-phthaldialdehyde; OPO, 1,3-dioleoyl-2-palmitoylglycerol; ORA, overrepresentation analysis; ORAC, oxygen radical absorption capacity; PAHs, polycyclic aromatic hydrocarbons; PAR, photosynthetically active radiation; pAUC, partial area under the curve; PBS, phosphate buffer solution; PC1, principal component 1; PC2, principal component 2; PCA, principal component analysis; PFTBA, perfluorotri-*n*-butylamine; pI, polarity index; PITC, phenylisothiocyanate; PL, phospholipid; PLS, partial least squares; PLS-DA, PLS discriminant analysis; Q, single quadrupole; QCs, quality control sample; QqQ, triple quadrupole; QTOF, quadrupole time-of-flight; RI, retention index; ROC, receiver operating characteristic***;*** RP, reversed-phase; RP-HPLC, reverse phase

high-performance liquid chromatography; RSD, relative standard deviation; RSM, response surface methodology; RT, retention time; SC, supercritical; SC-CO_2, supercritical carbon dioxide; SDS PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SE, Soxhlet extraction; SEA, set enrichment analysis; SELDI, surface-enhanced laser desorption/ionization; SEM, scanning electron microscopy; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; SHLE, superheated liquid extraction; SHWE, superheated water extraction; SP, sample preparation; SPE, solid-phase extraction; SPME, solid-phase microextraction; SRM, selected reaction monitoring; TBDMS, *tert*-butyldimethylsilylation; TC, tetracycline; TEAC, Trolox equivalent antioxidant capacity; TIC, total ion chromatogram; TLC, thin layer chromatography; TMCS, trimethylchlorosilane; TMPP, tris(2,4,6-trimethoxyphenyl)phosphonium; TMS, trimethylsilylation; TOF, time of flight; TPC, total phenolic content; TPP, three phase partitioning; TPS, trimethylsilyl propionate; UATPP, ultrasound assisted three phase partitioning; UHPLC, ultra-high-performance liquid chromatography; UHPSFC, ultrahigh performance supercritical fluid chromatography; US, ultrasound; USAE, ultrasound-assisted extraction; USAEE, US-assisted emulsification–extraction; USAEH, ultrasound-assisted enzymatic hydrolysis; USAL, ultrasound assisted leaching; USALLE, US assisted liquid–liquid extraction; UV, ultraviolet; VOC, volatile organic compound; VOO, virgin olive oil; W, watt; XRD, X-ray diffraction; Δ8-THC, δ8-tetrahydrocannabinol; Δ9-THC, δ9-tetrahydrocannabinol; Δ9-THCA, δ9-tetrahydrocannabinolic acid; Δ9-THCV, δ9-tetrahydrocannabivarin.

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